REMARKS

The Final Office Action of August 11, 2005 presents the examination of claims 6-11. These claims remain pending and are not further amended.

The sole ground of rejection is of claims 6-11 under 35 U.S.C. § 112, first paragraph, for alleged lack of enabling disclosure. In particular the Examiner asserts that the specification does not enable the making of the invention in that there is inadequate description of how to perform the step v) recited in claim 6.

The Examiner notes that step v) recites that cells of "whole organs" are to be added to the anti-idiotypic antiserum to form a suspension and that a supernatant should be allowed to separate from a sediment, which supernatant is the product antiserum that specifically binds to the antigen-stimulated lymphocytes. The Examiner takes a position that neither the specification nor the prior art provides any teaching as to what step v) is to accomplish, the kinds of organs needed to make the suspension, the amount of tissue to be used. The Examiner also asserts that no assay is described whereby a skilled artisan could determine any of these things.

Applicant submits that the specification provides enabling disclosure of the claimed invention. As it is step v) that is in question, Applicant's remarks are directed to the question of enablement of this step.

The question of enablement is addressed by whether undue experimentation is required to practice the claimed invention. The amount of experimentation is not determinative; considerable experimentation is not undue if guidance as to the experimentation to be performed is provided, and especially if the particular experimentation is expected by one of ordinary skill in the art.

Furthermore, the Examiner must distinguish between a situation in which a fair amount of work must be done, i.e. a mere technician must perform a number of well-known or otherwise

well-described steps, and a situation in which substantial experimentation must be done, i.e. someone must utilize inventive skill to resolve unanswered questions and make an invention operable. Applicant submits that the present rejection is a result of such confusing these two things and that, though some small amount of mere technical work might be needed to practice the invention, no inventive activity is needed to extend the teachings of the specification in order to practice the claimed invention.

A number of factors are to be evaluated in assessing whether experimentation needed to perform the invention is undue. See, *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988).

Accordingly, Applicant addresses each of the so-called "Wands" factors.

The nature of the invention

The claimed invention is a method for making an antiserum that specifically binds to antigen-stimulated lymphocytes. The invention also encompasses an antiserum made by such method and diagnostics utilizing the antiserum.

The making of polyclonal antisera by immunization of a mammal and isolating the serum from its blood is a mature art.

The breadth of the claims

The claims are not overbroad. The present claims recite serial immunization of rats. The step in contest at the moment recites that cells of whole organs of a rat of the same genetic line as that initially immunized are suspended, contacted with serum from the immunized animal and then allowed to settle out of a suspension.

The level of ordinary skill in the art

The level of ordinary skill in the art of antiserum production is generally considered to be high. One developing immunoassays in the cancer field, and reagents such as an antiserum to use in them, would generally hold a doctoral degree, and perhaps both a Ph.D. and an M.D.

The quantity of experimentation needed

The quantity of experimentation needed to practice the instant invention is not high. The particular step at issue is one in which normal tissues of an animal are used to absorb out from an antiserum antibodies that bind to such normal tissues. The experimentation necessary, if wholly unguided, to determine what tissues might be added to the absorption step, and the amount, would be to run a titration experiment using suspensions of cells taken from all, or selected combinations of tissues of a rat. Thus, perhaps a few dozen absorptions would need to be performed. The absorptions would have to be followed by a test for efficacy. The specification describes an agglutination test as a diagnostic test, and furthermore that the desired antigen is expressed in embryos, but not in normal tissues. Thus, the efficacy of the absorption would be tested by agglutination of the antiserum using suspension of embryonic tissues versus suspension of the normal tissues of a rat (i.e. the animal immunized to raise the antiserum).

Applicant notes that, as explained below, the specification indicates that absorption of the antiserum should be performed using at least kidney, liver and lung tissues. Thus, there is at least guidance as to the tissue types to be used and there remains merely the matter of titration of the amount. Furthermore, these procedures are more in the nature of mere technical work than experimentation.

Applicant submits that, in either case, such a quantity of experimentation is not large.

The guidance provided by the specification

The antiserum of the invention is described in the specification as one that binds to the idiotype of a T-cell receptor for a tumor antigen, which tumor antigen is expressed in embryonic

cells, but not in the normal tissues of an adult. (See page 3, lines 14-16, of the specification.) Relevant to the step v) in dispute, the specification at page 2, lines 3-7 describes that there are "heterospecific antigens" present in normal tissues as well as in tumor cells. These heterospecific antigens, indicated as leading to inaccurate diagnoses, are further said to be present in kidney, lung and liver tissues. (See page 2, lines 3-7, of the specification.)

The inclusion of a working example

There is a working example of the making and using of the invention that is commensurate in scope with the present claims.

The working example includes assay, using rat antiserum of the invention, of a sample from a human patient presenting a malignant tumor (rectal carcinoma), demonstrating that the antiserum shows a value in the assay in excess of the criterion value of 1.5. On the other hand, assay of a sample from a human patient presenting a benign tumor (fibroma) provides a result less than the criterion of 1.5. Furthermore, there is description of clinical results obtained in populations of patients presenting with malignant tumors, i.e. a "positive" population, and with various non-cancerous conditions, i.e. a "negative control" population. (See pages 7-8 of the specification.) Thus, contrary to the assertion of the Examiner, the specification provides an assay for a functional antiserum.

The state of the art at the time the invention was made

The state of the art at the time the invention was made was such that the production of polyclonal antisera by immunization of a mammal was a well-established procedure. One of ordinary skill in the art, at the time the invention was made, would have known that a polyclonal antiserum would contain numerous various antibodies, including undesired antibodies that would bind antigens other than a desired diagnostic antigen as in the present invention. The skilled artisan would further have known that such undesired antibodies could be removed from an antiserum by absorption of them from the antiserum using an insoluble substance, such as a suspension of tissue comprising cells expressing the antigens recognized by the undesired

antibodies. The amounts of such absorbents to be used would plainly be ones that would be in excess of that needed to accomplish the absorption.

The state of the art with respect to these aspects of preparing a polyclonal antiserum is evidenced by the following publications:

Exhibit 1, Yoshinaga et al, *Biology of Reproduction* 6:51 (1972), see esp. p. 52;

Exhibit 2, Allen et al., *Blood* 61:803 (1983), see esp. p. 803;

Exhibit 3, Ro et al., *J. Biochem. Biophys. Methods* 28:155 (1994), esp. pp. 156-157;

Exhibit 4, Dresse et al., "Immunization of Experimental Animals", pp. 8.1 ff in Handbook of Experimental Immunology Vol 1: Immunochemistry, 4th ed., E.M. Weir, editor, c. 1986 by Blackwell Scientific Publications, Oxford, see esp. p. 8.12.

Yoshinaga et al. describe the production of antiserum specific to uterine deciduoma antigens utilizing absorption of the antiserum with uterine tissue to remove undesired background antibodies binding to antigens presented by uterine tissue. Allen et al. sought antiserum specific for antigens presented in the membrane of erythrocytes; they describe absorption with red blood cells (to obtain a serum specific for antigens of the inner membrane ("ghosts") of erythrocytes), and with various leukemic cell lines representing different hematopoietic lineages, lymphocytes (white blood cells) and platelets. Ro et al. sought to improve antisera used for screening of recombinant *E. coli* for production of foreign antigens, and describe that absorption with extracts from frozen/thawed/sonicated *E. coli*, and/or boiled *E. coli* improves the result obtained compared to absorption with whole bacteria. Dresse et al. provides a chapter in a general laboratory manual and describes that antisera specific for thymocyte alloantigens can be prepared by absorption with liver tissue and erythrocytes. Thus, it is plain that one of ordinary skill in the art very well knows how to perform absorption procedures to remove unwanted antibodies against 'background" antigens from a polyclonal antiserum.

Applicant submits that, in view of the state of the art and the disclosure of the specification that antibodies against antigens presented by normal tissues of the animal should be removed from the antiserum of the invention, one of ordinary skill in the art is well-informed that a suspension of all of the normal tissues, or at least kidney, liver and lung tissue of an animal should be prepared and used as an absorbent. Applicant further submits that the skilled artisan would have known from the state of the art that the absorbed antiserum could be tested for specificity against embryonic antigens by tests such as an agglutination test using embryonic cells versus a suspension of cells used to perform the absorption step, to determine if the absorption had been effective.

The predictability in the art

Applicant concedes that the precise composition of a polyclonal antiserum, in terms of the particular antibodies that will constitute it, is unpredictable. However, given immunogenicity of the antigen, which is not challenged by the Examiner in the present case, and which anyway is demonstrated by the working example in the specification, it is very predictable that a polyclonal antiserum will contain a number of antibodies specific for the antigen used to raise the serum in a mammal. It is furthermore predictable that the specificity of a polyclonal antiserum for binding to the desired antigen target can be improved by absorption of the antiserum with an insoluble form of undesired antigens and precipitation of the (undesired)antigen-antibody complexes from the antiserum.

Finally, Applicant provides Exhibit 5, an abstract published in the program of the 1999 meeting of the American Society of Clinical Oncology by Berlin et al. The Berlin abstract summarizes clinical results obtained during testing of the invention to prove its clinical efficacy. The method of the invention was able to detect malignant lung tumors in about 88% of cases and to distinguish malignant from benign lung tumors with about 90% specificity. Applicant submits that these data firmly establish the operability of the invention.

The present application well-describes and claims patentable subject matter. The favorable action of allowance of the pending claims and passage of the application to issue is respectfully requested.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Mark J. Nuell (Reg. No. 36,623) at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

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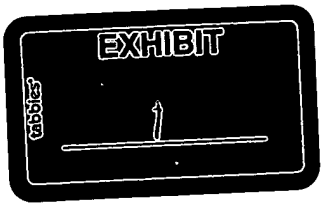
Respectfully submitted,

By _____

Mark J. Nuell, Ph.D.
Registration No.: 36,623
BIRCH, STEWART, KOLASCH & BIRCH, LLP
8110 Gatehouse Road
Suite 100 East
P.O. Box 747
Falls Church, Virginia 22040-0747
(703) 205-8000
Attorney for Applicant

Attachments: Exhibits 1-4



BIOLOGY OF REPRODUCTION 6, 51-57 (1972)

Rabbit Antiserum to Rat Deciduoma

K. YOSHINAGA

Laboratory of Human Reproduction and Reproductive Biology and Department of Anatomy, Harvard Medical School, Boston, Massachusetts 02115

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Antiserum to rat deciduoma was produced in the rabbit. The antiserum had an inhibitory effect on deciduoma formation when injected into the uterine lumen of pseudopregnant rats immediately after traumatization of the uterus. Normal rabbit serum had no inhibitory effect. The antiserum after absorption with the extract of immature rat uterus indicated the presence of a component specifically reactive to the deciduoma extract by an agar gel double diffusion test. This was shown by a precipitin line between deciduoma extract and the antiserum from which nonspecific antibodies to rat uterine components had been removed. The antiserum after absorption with the extract of immature rat uterus inhibited deciduoma formation when given locally into the uterine lumen.

Application of immunological principles and techniques to the study of reproductive biology has become progressively important. Specific neutralizing and binding ability of antibodies has been utilized for measurement of small quantities of protein and steroid hormones (cf. Diczfalusy, 1969; Péron and Caldwell, 1970). This ability is also utilized for the study of roles of hormones in various physiological phenomena (Madhwa Raj and Moudgal, 1970; Talaat and Laurence, 1969; Loewit, Badawy and Laurence, 1969; Yoshinaga, Moudgal, and Greep, 1971).

Rat decidual tissue arises from the endometrial stromal cells during the course of ovoimplantation. It serves as a source of nutrition, limitation of trophoblastic invasion, isolation of each embryo, and formation of a cleavage zone for placental separation at the time of delivery (De Feo, 1967). Deciduomal tissue is produced by traumatizing the endometrium of rats during the sensitive stage of pseudopregnancy as well as under proper hormonal conditions such as lactation. It is essentially identical to decidual tissue except that its formation is evoked by artificial means (Selye and McKeown, 1935; Krehbiel, 1937). It was of interest to examine whether it was possible to produce biologically potent antiserum against deciduoma by routine immunological procedures. This report describes such a trial and the effects of the antiserum on the formation of deciduomata in pseudopregnant rats.

MATERIALS AND METHODS

Preparation of antigen. Adult female Charles River CD rats were used for production of deciduomata. The estrous cycles were traced by vaginal smear and the animals were made pseudopregnant by applying mechanical stimulation to the uterine cervix with a glass rod on the day of estrus. The last day of vaginal cornification was designated as Day 1 of pseudopregnancy. The animals were laparotomized on Day 5 and both uterine horns were traumatized by scratching the endometrium with a curved needle along the entire uterine horn. On Day 9 the rats were killed and uteri were dissected out. The uterine horns were split longitudinally and deciduomal tissue was separated from the myometrium by scraping with the sharp edge of a scissor. Blood on the tissue was blotted as much as possible by rolling it on a sheet of sterile paper. Tissues were pooled in a Petri dish placed in ice. After recording the wet weight, the tissues were homogenized in cold saline in a glass homogenizer cooling with iced water. The final concentration of homogenate was made in such a way that each milliliter contained 250 mg of the tissue. The deciduomal homogenate was either used immediately after preparation or kept frozen until use.

Preparation of antiserum. The tissue homogenate was injected to young adult female New Zealand

white rabbits intramuscularly in both hind legs, and intraperitoneally. Each rabbit received 500 mg tissue homogenate in 2 ml saline as a sensitization injection. One week later the rabbits were injected with the homogenate (500 mg) emulsified in 2 ml of Freund complete adjuvant (Difco). This immunizing injection was repeated every week. One week after the 6th injection the animals were bled by puncture of the ear artery. After confirming the serum had antibodies to the deciduomal tissue extract by an agar gel double diffusion test, the animals were maintained on 3-week rotation schedule of booster shot, and bleeding with intervals of one week between booster shot and bleeding, and two weeks between bleeding and the next booster shot. The serum was separated from collected blood after clot formation by centrifugation, and divided into 5–10 ml aliquots. As a preservative Merthiolate Sodium ([(o-Carboxyphenyl)thio]ethylmercury sodium salt) was added to the serum to a final concentration of 1:10,000, and kept at 4 C. Normal rabbit serum collected from young adult rabbit was treated in the same fashion as was the antiserum.

Biological test of the antiserum. Since the biological potency of the antiserum was the first concern in the study, the prepared antiserum was tested for its inhibitory effect on deciduoma formation in the rat. The deciduoma antiserum (DAS) was administered subcutaneously on Day 5, or intraperitoneally on Day 5 or 6 of pseudopregnancy, or locally into the uterine lumen on Day 5 after traumatization of the uterus. The rats were made pseudopregnant by cervical stimulation, the uterus was traumatized on Day 5, and the animals were killed on Day 9. In the experiments wherein DAS was given systemically, traumatization of the uterus was applied on one horn by scratching the endometrium with a curved needle, whereas the uterine horns were pinched with a pair of serrated forceps along the entire uterine horn in the experiments where DAS was given locally into the uterine lumen. The local injection of the serum was performed as follows: A 25-gauge needle was inserted into the uterine lumen at a point close to the cervix, a ligature of 3-0 silk was applied over the uterine horn at the junction of the two horns and tightened with the needle inside. The serum (0.1 or 0.05 ml) was injected into the lumen and the ligature was tightened as the needle was withdrawn. DAS was injected into the right horn and the same volume of normal rabbit serum (NRS) was injected into the left horn. At autopsy the uterine horns were dissected out and examined macroscopically for deciduoma. After the weight of each horn was recorded a portion of the horn was fixed in Bouin's solution for further histological study. The tissues were embedded in paraffin, sectioned at 8 μm and stained with hematoxylin and eosin.

Absorption of nonspecific antibodies to rat uterine tissue. Nonspecific antibodies were absorbed from the antiserum by precipitation with a saline extract of immature rat uterus (RUE), which was prepared as follows: Uteri of 40-day-old rats were homogenized in cold saline. Concentration of the homogenate was made to 200 mg/ml. The homogenate was then centrifuged at 4 C at 3,000 rpm for 30 min. Supernatant was separated and Merthiolate was added (1:10,000) and kept at 4 C. Deciduomal extract (DE) was similarly prepared from the homogenate which was used for immunization of the rabbits. RUE was added to DAS every day until precipitation was no longer formed after addition of RUE for at least 24 hr. The serum was tested at various stages of absorption on an Ouchterlony agar gel double diffusion plate for their reactivity with RUE and DE.

Biological activity of DAS after absorption by RUE. Since a specific antibody to DE was shown not to be absorbed by RUE, and a preliminary biological test showed that 0.1 ml of absorbed DAS did not inhibit deciduoma formation when administered into the uterine lumen, the absorbed DAS was lyophilized and the concentrated DAS was tested for its inhibitory effect on deciduoma formation. Though the volume of the absorbed DAS injected into the uterine lumen was 0.1 ml, it was equivalent to 0.13 and 0.33 ml of the original DAS. Similarly lyophilized NRS of the same concentrations were administered in the control horn.

RESULTS

Immunological reactivity of DAS with RUE and DE. From the number of precipitin lines which showed reactivity of identity and appearance common to RUE and DE it can be assumed that these two extracts share a minimum of three common antigens. Absorption of the antiserum with RUE further indicated the presence of a component specific to DE (Fig. 1).

Effect of systemic injection of unabsorbed DAS. No inhibition of deciduoma formation was observed in 16 rats where 0.5 ml unabsorbed DAS had been injected systemically. Traumatization of the uterus resulted in massive deciduoma; the uterine horn weight was approximately ten times that on the intact side. No significant differences

were detected between any of the groups listed in Table 1.

Effect of local injection of unabsorbed DAS. Failure of inhibition of deciduoma formation by systemic injection of unabsorbed DAS led us to examine whether the amount of antibodies injected was not enough to reach the uterus to exert its inhibitory effect. To test this possibility the antiserum was injected into the uterine lumen. The results are summarized in Table 2. Inhibition of deciduoma formation was observed in the uterine horn segment where 0.1 ml unabsorbed DAS had been injected intraluminally. On the other hand no inhibition was observed in the segment where NRS had similarly been injected (Fig. 2). Average weight of the DAS treated horn was 8–40% of that of NRS treated horn. The inhibitory effect of DAS was limited within the uterine horn, and deciduomal response was always observed between the ligature and the cervix on the same horn. When 0.05 ml of unabsorbed DAS was injected the inhibitory effect was less marked, inhibition being observed in 4 out of 8 rats thus treated.

Effect of local injection of absorbed DAS. Deciduoma formation was inhibited in all rats when they were injected intraluminally with absorbed serum equal to 0.33 ml of original DAS. Whereas, no inhibitory effect was observed in the animals treated with 0.13 ml eq judging from the uterine horn weight (Table 2). Thus antibodies specific to deciduoma were still present after absorption of DAS with extract of immature rat uterus. Local inhibition of deciduoma formation with DAS after absorption with uterine extract is shown in Fig. 3. The uterus shows a response similar to that observed in rats treated with unabsorbed DAS. However, the inhibitory effect was not as strong as that of unabsorbed DAS and small lumps of deciduoma were occasionally observed in some animals.

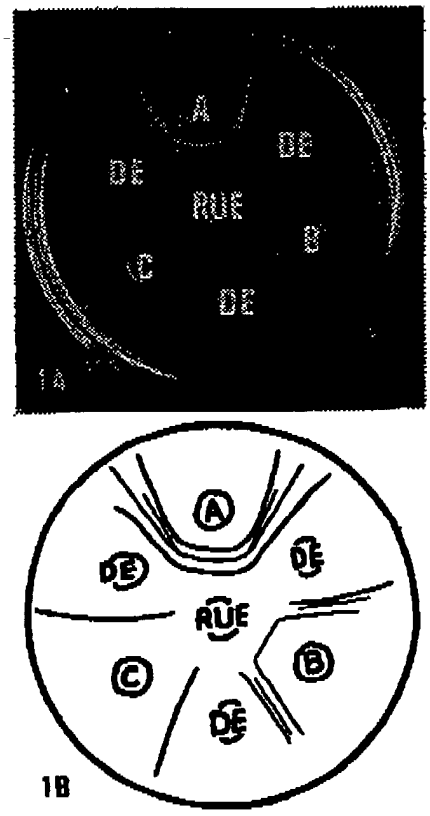


FIG. 1. Immological reactivity of the deciduoma antiserum with rat uterine extract and deciduoma extract. RUE (rat uterine extract) was placed in the center well. Deciduoma antiserum was placed in wells A, B, and C at various stages of absorption with RUE. A: unabsorbed antiserum, B: partially absorbed, and C: completely absorbed antiserum. From the number of precipitin lines which showed reactivity of identity and appearance common to RUE and DE, these two tissue extracts appear to share a minimum of three common antigens (between A and RUE; A and DE). Partial absorption reduced the number of lines (B), and complete absorption indicated the presence of a component specific to DE (C).

TABLE 1

EFFECT OF 0.5 ML UNABSORBED DECIDUOMA ANTISERUM GIVEN SYSTEMICALLY ON DECIDUOMA FORMATION

	Route of injection	Day of injection	No. of rats	Uterine horn weight		
				Traumatized	Control	Ratio (T/C)
DAS[a]	ip	5	5	1113.2 ± 211.9[b]	118.4 ± 17.1	9.6 ± 2.2
DAS	ip	6	4	1345.8 ± 161.5	135.0 ± 33.0	10.3 ± 1.7
DAS	sc	5	4	1505.0 ± 176.2	132.0 ± 18.0	11.5 ± 0.8
NRS	ip	5	4	1518.0 ± 501.7	140.5 ± 19.3	10.7 ± 2.2

[a] DAS = deciduoma antiserum, NRS = normal rabbit serum.
[b] Mean ± SD.

TABLE 2

INHIBITORY EFFECT OF INTRALUMENAL INJECTION OF DAS ON DECIDUOMA FORMATION IN PSEUDOPREGNANT RATS

Sera injected		No. of rats	No. of rats with deciduoma inhibition	Uterine horn weight (mg)[a]			P "t test" (c) vs. (d)
(a) Volume instilled into the uterine lumen (ml)	(b) Original volume of sera in (a) (ml)			DAS-treated (c)	NRS-treated (d)	d/c	
Unabsorbed							
0.1	0.09	7	7	361.5 ± 128.0[b]	1238.0 ± 177.9	3.76 ± 1.30	<.01
0.05	0.045	7	4	717.2 ± 366.1	1080.0 ± 354.5	1.85 ± 1.22	NS
Absorbed with immature rat uterine extract							
0.1	0.33	5	5	381.8 ± 184.3	739.2 ± 237.8	2.52 ± 0.78	<.05
0.1	0.13	5	0	850.0 ± 73.0	970.6 ± 164.6	1.14 ± 0.17	NS

[a] The uterine horns were dissected at the ligature applied close to the two horns and at the uterotubal junction.
[b] Mean ±SD.

Histological observations. A typical deciduoma formation was observed in all the rats treated with NRS. The decidual cells located antimesometrially, had dense cytoplasm and their nuclei were large and oval shaped. Typical binucleated cells were abundant in this region and occasional mitotic figures were observed (Fig. 5). The epithelial cells bordering the uterine lumen were flat and degenerating. The mesometrial decidual cells had round but slightly smaller nuclei with a relatively small amount of cytoplasm. The intercellular spaces were prominent. The epithelial cells bordering the lumen were cuboidal.

In the uterine horns treated with unabsorbed DAS deciduoma formation was obviously inhibited as judged by the appearance and weight of the uterine horn, and confirmed microscopically by lack of deciduoma in the endometrium. The antimesometrial portion of the endometrium is shown in Fig. 4. Unlike the decidualized horn, the epithelial cells bordering the lumen were columnar and intact. Subepithelial stroma was characterized by a loose connective tissue and stromal cells with small nucleic and little cytoplasm. This zone was surrounded by another zone in which stromal cells were more densely packed. Between

FIG. 2. Local inhibition of deciduoma formation by unabsorbed deciduoma antiserum. DAS (0.1 ml) was injected into the right horn on Day 5 of pseudopregnancy immediately after traumatization of the uterus. NRS (0.1 ml) was injected into the left horn. The uterus was dissected out on Day 9. Note the lack of deciduoma on the horn treated with DAS above the ligature.

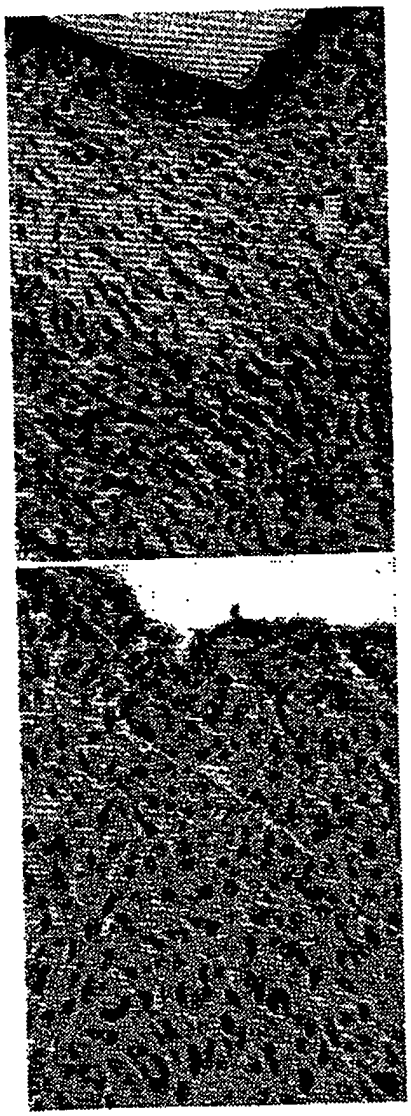
FIG. 3. Local inhibition of deciduoma formation by absorbed DAS. Absorbed DAS (right), and NRS (left) were injected into the uterine horns on Day 5 of pseudopregnancy. Inhibition of deciduoma formation can be seen in the horn treated with DAS. Small masses of deciduomal tissue were observed in the portion close to the ligature in DAS treated horn.

this zone and the circular muscle were stromal cells with round nuclei and relatively dense cytoplasm. In the horns treated with 0.33 ml eq absorbed DAS a similar picture was observed in the portion of the uterus without lumps. In the portion with a lump a small mass of deciduoma was observed in the antimesometrial side of the endometrium.

DISCUSSION

An earlier trial to produce antisera to rat deciduoma by Bockman and DeFeo (1964) failed to show the presence of the specific antigen in deciduoma. The present study shows that it is possible in the rabbit to produce antibodies specific to rat deciduoma potent enough to inhibit deciduoma formation. Deciduomal tissue, therefore, contains antigen(s) immunologically distinguishable from that in the uterus of immature rats. In a separate experiment the antiserum was absorbed with a saline extract of rat uterus harvested on Day 5 of pseudopregnancy, and tested on a double diffusion plate, the serum still reacted with DE. A precipitin line produced between DE and DAS absorbed with pseudopregnant rat uterine extract was identical with that produced between DE and DAS absorbed with immature rat uterine extract (Yoshinaga, unpublished observation). This result justifies the use of immature rat uterus extract for absorption of nonspecific antibodies to rat uterus. The results of agar gel double diffusion test for reactivity between unabsorbed DAS and DE or RUE showed that at least three different antibodies reacted to antigens which were common to DE and RUE. This is to be expected when we recall that deciduomal cells originate from the endometrial stromal cells. Identification of these antigenic constituents of the uterus and deciduomal tissue remains to be studied further.

Failure of inhibition of deciduoma forma-



tion by systemic administration of 0.5 ml unabsorbed DAS appears to be due to low antibody titer. It is possible to inhibit the deciduoma formation by systemic administration of a larger dose of DAS since inhibition was observed in two rats in a preliminary study using the antiserum from a rabbit which showed a higher antibody titer, but unfortunately died before completion of the work. Antisera produced against placental tissue have been reported to interrupt pregnancy by systemic administration in the rat (Seegal and Loeb, 1940), mouse (Koren, Abraham and Behrman, 1968), and guinea pig (Cohen and Nedzel, 1940).

The inhibitory effect of the absorbed DAS given locally demonstrates that the antibodies to deciduomal tissue were potent after removal of nonspecific antibodies to the uterine tissue. However, the potency of the absorbed DAS was weaker than that of unabsorbed DAS. It appears likely that the unabsorbed DAS exerted its inhibitory action not only on the decidualized cells but also on the other components of the uterus including the endometrial stromal cells from which deciduomal cells originate. Fractionation, isolation, and purification of the antiserum for further study of the inhibitory mechanism of decidualization is in progress.

ACKNOWLEDGMENTS

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FIG. 4. Cross sections of the antimesometrial side of the endometria (×400). Sections were made at 8 μm and stained with hematoxylin and eosin. Unabsorbed DAS-treated horn. Note the absence of decidual cells. Epithelial cells are intact and stromal cells have dense nuclei and small amount of cytoplasm.

FIG. 5. NRS-treated horn. Note the epithelial cells are flat and degenerating. The endometrium is occupied by decidual cells which are characterized by their large nuclei, occasionally binucleated, and by their rich cytoplasm.

were extremely helpful in carrying out this work. Technical assistance by Miss C. J. Staley is appreciated.

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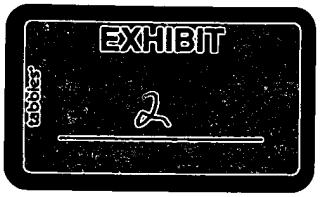

CONCISE REPORT

Reorientation of Membrane Polypeptides During Erythrocyte Maturation

By Robert W. Allen and Beverly A. Hoover

Messenger RNA extracted from the erythroleukemic cell line K562 was translated in vitro and the translation products reacted with an antiserum raised against human erythrocyte ghosts. Polypeptides immunoprecipitated by the antiserum were characterized by SDS-polyacrylamide gel electrophoresis and fluorography. The antiserum immunoprecipitated polypeptides with nominal molecular weights of 37,000 (p37), 20,000 (p20), 19,000 (p19), 18,000 (p18), 14,000 (p14), 13,000 (p13), and 11,000 (p11) daltons. Since the antiserum was raised against antigenic determinants present on both the inner and outer surface of the red cell membrane, differential absorption of antiserum with intact red cells, or ghosts, was used to localize the translation products to the inner or outer membrane surface. Absorption was also used to determine if any of the immunoprecipitated translation products represented membrane markers for the erythroid lineage. Absorption of the antiserum with red cell ghosts removed all antibodies reacting with in vitro translation products. Absorption with intact cells from various lineages removed anti-p20 antibodies and did not absorb anti-p19 or anti-p18 antibodies. Absorption with intact cells from all lineages except mature erythrocytes absorbed anti-p37, anti-p14, and anti-p13 antibodies, suggesting that these antigens are expressed on the outer membrane surface. Mature erythrocytes were incapable of absorbing these antibody populations, suggesting a lineage-specific reorientation of these antigens in the membrane during erythropoiesis.

THE CENTRAL involvement of cell membrane components in many aspects of blood cell development and eventual function has been amply demonstrated. In each of the hemopoietic lineages, membrane proteins with restricted expression to a particular lineage have been identified, and in many cases, characterized biochemically.[1-3]

In the erythroid lineage, the composition of the cell membrane has been extensively analyzed both biochemically and immunologically.[5,6] It appears that most, if not all, of the membrane proteins display asymmetry in the membrane in that they are exclusively localized to the extracellular or intracellular face of the membrane.[6]

Studies on the synthesis and processing of red cell membrane proteins has been hindered by the lack of active protein synthetic machinery in mature red blood cells. Permanent leukemic cell lines derived from erythroid progenitors have thus proven useful in studies on gene expression and protein synthesis during erythropoiesis.[9-12] The human K562 erythroleukemic cell line, for example, has been used in studies on the regulation of hemoglobin gene activity[13-17] as well as on the biosynthesis of membrane proteins.[18-20] We have used the K562 cell line as a source of mRNA coding for red cell membrane proteins. Several polypeptides synthesized from K562 mRNA were immunoprecipitated with an antiserum raised against red cell ghosts. The assignment of these antigens to the inner or outer membrane surface of various hemopoietic cell types was determined by differential absorption of the antiserum followed by immunoprecipitation.

MATERIALS AND METHODS

Cell Lines and Maintenance

The leukemic cell lines used in this study included Victor and Molt-4, representatives from the B- and T-lymphoid lineages, respectively, and K562 cells, a line displaying characteristics of the erythroid lineage.[10,12,19] All lines were cultured at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum (K.C. Biological, Lenexa, Kans.) in a humidified atmosphere of 5% CO_2 and 95% air.

Extraction of RNA, In Vitro Translation, and Immunoprecipitation

RNA was extracted from K562 cells using the guanidine-HCl procedure of Cox.[21] Total RNA was translated in the wheat germ system essentially as described by Roberts and Patterson[22] using ^{35}S-methionine (New England Nuclear, Boston, Mass.) as the incorporated labeled amino acid. Translation products were immunoprecipitated with antiserum raised against human red cell ghosts prepared according to Dodge et al.[23] The immunoprecipitation procedures have been described.[18]

Antiserum Absorption

One-hundred microliters of antiserum diluted 1:10 with PBS + 2 m*M* PMSF were mixed with an equal volume of the various cell types or red cell ghosts for 2 hr on ice. The cells (or ghosts) were centrifuged and the supernatant stored at −20°C until used.

From the Department of Research, American Red Cross Blood Services, Missouri-Illinois Region, St. Louis, Mo.

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Address reprint requests to Dr. Robert W. Allen, Department of Research, American Red Cross Blood Services, Missouri-Illinois Region, 4050 Lindell Boulevard, St. Louis, Mo. 63108.

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RESULTS

One-hundred microliter wheat germ translations programmed with 160 μg/ml of total K562 RNA routinely yielded 10^6 cpm of TCA-precipitable protein. When these total translation products were reacted with excess amounts of rabbit antibodies raised against red blood cell ghosts, about 0.1%–0.5% of the total radioactivity was immunoprecipitated. The immunoprecipitates consisted of polypeptides with nominal molecular weight of 37,000 (p37), 20,000 (p20), 19,000 (p19), 18,000 (p18), 14,000 (p14), 13,000 (p13), and 11,000 (p11) daltons (Fig. 1A, lane A, Fig. 1B, lane B). Of the polypeptides immunoprecipitated, p37 contained the greatest radioactivity. Variations in the amount of antiserum added to the translations did not significantly alter the intensity of any band in the fluorograms, suggesting that variations in antibody titer do not account for the differing polypeptide band intensities. These results suggest that either p37 contains a higher methionine content than the other polypeptides immunoprecipitated or that its mRNA is more abundant in the cells.

The antiserum was raised against membrane antigens exposed on both the inner and outer surface of red cells. Through differential absorption of the antiserum with intact cells or ghosts, it should be possible to localize the antigenic determinant(s) present on the polypeptides translated in vitro to the inner or outer surface of the cell membrane. Aliquots of antiserum were absorbed with a variety of cell types or with ghosts and then reacted with translation products programed with K562 mRNA. Absorbing the antiserum with the Victor (B-lymphoid), Molt-4 (T-lymphoid), or K562 (erythroid) cell lines removed antibodies reacting with p37, p20, p14, and p13 (Fig. 1A, lanes B and D). Absorbing the antiserum with peripheral blood lymphocytes or platelets yielded comparable results (Fig. 1B, lane C). These results indicate that the antigenic determinants located on the p37, p20, p14, and p13 polypeptides are expressed on the outer cell surface in these cell types. Absorption of the antiserum with these cell types did not noticeably remove antibodies reacting with p19, p18, or p11, suggesting that these antigenic sites are either exposed on the inner membrane surface or the polypeptides are not synthesized in the cells (Fig. 1A, lanes B and D, Fig. 1B, lane C). It should be recalled, however, that K562 cells contain these messenger RNAs and presumably the polypeptides.

Absorption of the antiserum with intact red cells removed antibodies to p20 and did not remove antibodies to p19 and p18, as had been observed for absorption with the other hemopoietic cells (Fig. 1A, lane C). Intact red cells absorbed antibodies reacting with p11 and, more interestingly, did not absorb antibodies reacting with p37, p14, and p13 (Fig. 1A, lane C). Absorption of the antiserum with red cell ghosts or with ghosts that had been washed with 0.5 *M* NaCl absorbed antibodies to all the polypeptides (Fig. 1B, lanes D and E). These results suggest that the antigenic determinants on the p37, p14, and p13 polypeptides are expressed on the inner, rather than the outer, surface of mature red cells.

The small amount of residual antibodies reacting with p37 left in the absorbed antiserum samples are probably directed against antigenic determinants on that portion of the polypeptide buried in the cell membrane and therefore not exposed for interaction with antibody.

DISCUSSION

The antiserum used in the study was prepared against red blood cell ghosts prepared according to Dodge et al.[9] and contained antibodies to antigenic determinants expressed on both sides of the cell membrane. The antiserum immunoprecipitated several polypeptides from in vitro translations programmed with K562 mRNA. Through differential absorption of the antiserum with intact hemopoietic cells or red cell ghosts, it has been possible to localize the antigens translated in vitro to the inner or outer surface of the membrane. The p20, p19, and p18 polypeptides were found to be expressed in the same way in all the hemopoietic cells examined, i.e., p20 is expressed on the outer membrane surface, while p19 and p18 are not expressed on the outer surface. The p37, p14, and p13 polypeptides are expressed on the outer surface of all hemopoietic cells examined except erythrocytes. In erythrocytes, these antigens appear to be reoriented such that they are expressed on the inner membrane surface. Erythrocytes alone appear to express p11 on the outer cell surface.

The results suggest that the orientation of membrane proteins in hemopoietic cells can differ in a lineage-specific manner. It is especially interesting that K562 cells expressed the p37, p14, and p13 antigens in a manner that differs from mature red cells. K562 cells are known to express the erythroid differentiation markers, hemoglobin and glycophorin, and the line is generally considered to have originated from an erythroid progenitor cell type.[10–13,19,20] If K562 cells reflect a progenitor cell phenotype in the erythroid lineage, then the reorientation of the p37, p14, and p13 polypeptides must occur in the lineage at a maturation step later than the K562 phenotype.

The reorientation of the membrane proteins could serve an important role in blood cell development. If polypeptides such as p37, p14, and p13 serve as

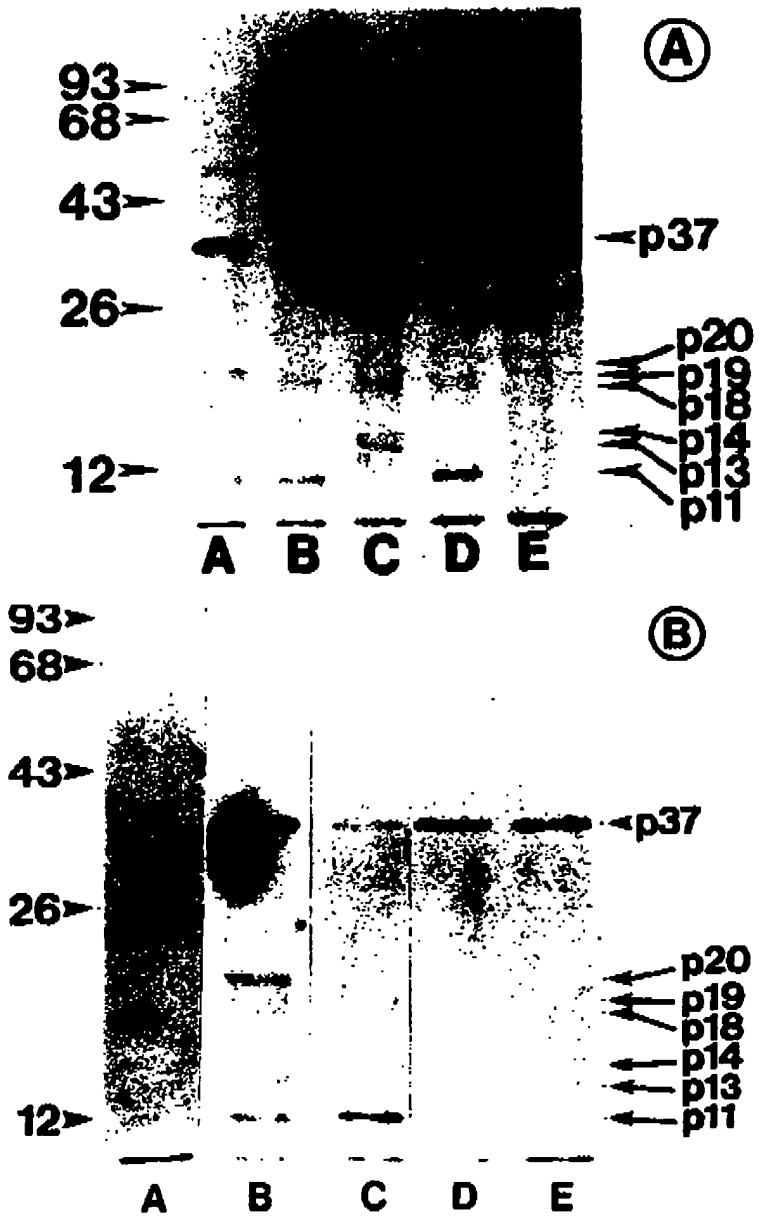

Fig. 1. Immunoprecipitation of K562 mRNA-directed translation products with antiserum absorbed with various hemopoietic cells. K562 mRNA was translated as described in Materials and Methods. Products from 100 μl of translation were reacted with unabsorbed antiserum or antiserum absorbed with the different hemopoietic cells and subsequently processed for SDS-PAGE and fluorography as described in Materials and Methods. (A) Products immunoprecipitated with: (lane A) unabsorbed antiserum; (lane B) antiserum absorbed with K562 cells; (lane C) antiserum absorbed with erythrocytes; (lane D) antiserum absorbed with Victor or Molt-4 lymphoblastoid cells; (lane E) preimmune rabbit serum. (B) Products immunoprecipitated with: (lane A) preimmune rabbit serum; (lane B) unabsorbed antiserum; (lane C) antiserum absorbed with platelets or peripheral blood lymphocytes; (lane D) antiserum absorbed with erythrocyte ghosts; (lane E) antiserum absorbed with erythrocyte ghosts previously incubated 15 min at room temperature with 0.5 M NaCl.

receptors for environmental stimuli, their reorientation in the membrane might make the cell unresponsive to such stimuli and signal a commitment step towards the erythroid lineage.

The polypeptides immunoprecipitated from the in vitro translations have not yet been identified within the framework of known red cell membrane proteins.[6,8] Membrane proteins translated in vitro can differ significantly from their processed counterparts in the cell membrane.[24,25] These differences are due primarily to a lack of glycosylation in vitro and the frequent presence of an extra 15–20 N-terminal amino acids residue constituting a signal sequence that is proteolytically cleaved from membrane proteins as they are inserted

into the membrane.[26] An example of how processing can greatly alter the characteristics of a membrane protein can be seen in the case of glycophorin. Gahmberg et al.[20] have reported that translation of glycophorin mRNA in vitro results in the synthesis of a polypeptide of approximately 19,000 daltons. The monomeric molecular weight of glycophorin isolated from red cells or K562 is approximately 39,000 daltons,[5] indicating that extensive glycosylation of the polypeptide has occurred during its synthesis and insertion into the membrane.

We are currently using the techniques of two-dimensional gel electrophoresis and peptide mapping to compare the in vitro translation products with membrane proteins extracted from K562 cells or erythrocytes. These studies should help identify the in vitro translation products and possibly clarify the development and/or physiologic consequences of their reorientation in the erythrocyte membrane.

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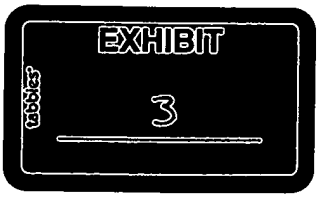

JBBM 1041

Short Note

Rapid purification of antiserum against *Mycoplasma hyopneumoniae* by an efficient absorption method

Lee-Hwa Ro [a], Re-Jen Chen [b], David Shiuan [b,*]

[a] *Division of Molecular Biology, Development Center For Biotechnology, Taipei, Taiwan, ROC*
[b] *Department of Biology and Institute of Life Sciences, National Sun Yat-sen University, Kaohsiung, Taiwan, ROC*

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Abstract

A simple and efficient method for the removal of unwanted cross-reactive antibodies has been developed. The antiserum purification method was based on treatment of the antiserum with both sonicated extracts and boiling extracts of the *Escherichia coli* host cells used in immunoscreening the lambda EMBL3 library. We have demonstrated unambiguously that through this simple treatment, the rabbit anti-*Mycoplasma hyopneumoniae* antiserum can be effectively purified so that the amount of antibodies cross-reacted with *Escherichia coli* lysate proteins is drastically reduced. Compared with the traditional absorption methods, which require the chemical coupling of an absorbing agent to an insoluble support, and affinity purification methods, which have harsh denaturing condition, this method should greatly facilitate a successful immunoscreening experiment.

Key words: *Mycoplasma hyopneumoniae*; Antiserum; Purification; Absorption; Immunoscreening

Mycoplasma hyopneumoniae is the etiologic agent of enzootic pneumonia of swine, a chronic nonfatal disease affecting pigs of all ages [1]. In spite of the fact that pneumoniae caused by *Myoplasma hyopneumoniae* is one of the most serious swine diseases, understanding its pathogenicity as well as the control of the disease by vaccination are still preliminary [2–5]. In addition, the rather small size of the genome and its unusually low GC content also prompted us to gain more insights into the structures and regulation patterns of its various genes. As a first step, we

* Corresponding author. Fax: + 886 7 5319333.

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studied genes coded for membrane surface proteins, which may constitute membrane structural proteins, receptor proteins, enzymes as well as cytopathic factors involved in the pathogenesis of this microorganism. However, the immunoscreening of the λEMBL3 library or λgt11 expression library for the surface protein genes was hampered by the severe cross-reaction of the rabbit anti-Mycoplasma antiserum with the *E. coli* proteins produced in large amount during the plague formation processes. Efforts to remove the cross-reacting antibodies by simple absorption [6,7] with *E. coli* cells or *E. coli* proteins coupled on a nitrocellulose membrane, affinity column or other solid support [2,8–13] were unsatisfactory. Therefore, a rapid and efficient purification method which is simple and less tedious than the current immunoprecipitation and immunoaffinity chromatographic methods is greatly needed [9–13].

1. Preparation of antiserum against *M. hyopneumoniae*

Rabbit anti-*M. hyopneumoniae* hyperimmune serum was prepared for immunoscreening [4,14,15]. The rabbit was immunized with five intramuscular injections of a mixture of equal amounts of Freund's complete adjuvant and immunogens. The immunogens were prepared from *M. hyopneumoniae* strain 232 [4] cultured in Friis broth medium supplemented with 20% (v/v) porcine serum, to stationary phase, concentrated 100 times and resuspended in Hank's balanced salt solution (Grand Island Biological) with 0.15% formalin. The 1.5 ml immunogen-adjuvant mixture was given at intervals of 3 days. Three weeks after the last intramuscular injection, the rabbit was boosted with three additional intravascular injections, each with 1.5 ml immunogen, at 5-day intervals. 1 week after the last injection, the animal was bled and antiserum was collected for immunoblot analysis.

2. Purification of antiserum against *M. hyopneumoniae*

Cross-reactions of antibodies with host cell proteins had presented a serious problem in immunoscreening experiments. Various methods, including absorption of the antiserum with plain *E. coli* cells, absorption of the antiserum with protein precipitated from *E. coli* lysate caused by phage lambda, and absorption of antiserum by *E. coli* proteins coated on nitrocellulose membrane or other solid supports, had been used to remove the cross-reactive antibodies from the antiserum used for immunoscreening. Although these methods have been widely used and are generally applicable, further improvement to guarantee a feasible immunoscreening process is still necessary. Therefore we have tried to develop a rapid and more efficient procedure that can be used rountinely.

The method involves absorption of the antiserum with the freeze-thaw-sonication extract and the boiling extract of *E. coli*. The *E. coli* P2392 cells from a 2 l overnight culture was harvested and resuspended in 20 ml of buffer containing 50 mM of Tris-HCl (pH 8.0) and 10 mM of EDTA. The sonicated extract was

prepared by three freeze (−20°C, 2 h) − thaw (room temperature, 0.5 h) cycles followed by sonication (4 min at 0°C, 8 × 30 s, 50% duty cycle, Heat System W350 sonicator with output energy of 80–100 W). Most cells were broken by this treatment as observed under a microscope. The boiling extract was prepared by resuspending the pellet of a 2 l overnight culture in 20 ml of deionized water in a 50 ml plastic conical tube, and placed in a boiling water bath for 10 min.

Absorptions with sonicated extract and/or boiling extract were performed by mixing 1 ml of the extract with 1 ml of rabbit antisera. After incubating overnight at 4°C, debris was removed by centrifugation (Eppendorf microfuge, 12000 rpm for 5 min). The purified rabbit antiserum were stored with 0.01% sodium azide at 4°C before immunoscreening experiments were carried out.

3. Immunoscreening with the purified antiserum

The removal of unwanted specificities is a determining step in the immunoscreening process. The absorption effects of the sonicated extract, the boiling extract and both extracts combined were examined by Western blot analysis [16]. As shown in Fig. 1, groups of total protein of *M. hyopneumoniae* (lane 1), *E. coli* total proteins (lane 2) and lambda lysate proteins (lane 3) were separated by SDS-polyacrylamide gel electrophoresis and blotted (Hoefer HE70 semi-dry blotter) onto NC (nitrocellulose) paper. Typically, a 1:1000 dilution of the absorbed antiserum with TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) was incubated with the blotted NC paper at room temperature overnight or 2 h at 37°C. After washing the filter three times for 10 min with TBS, the antibody probe was detected by alkaline phosphatase conjugated probes. The filter paper was incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (Boehringer-Mannheim) 1:3000 dilution in TBST with 5% powdered skim milk at room temperature for 2 h. The paper was washed several times with TBST (150 mM NaCl, 50 mM Tris-HCl, pH 8.1, 0.05% Tween 20) and once with TBS, and then equilibrated with 20 ml of buffer A (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). Visualization was performed by the addition of 45 ml freshly prepared NBT solution (Nitroblue tetrazolium salt 75 mg/ml in 70% (v/v) dimethylformamide) and 35 ml of X-phosphate solution (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt, 50 mg/ml in dimethylformamide). When the expected protein bands appeared, the reaction was stopped with 50 ml of buffer B (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

As shown in Fig. 1, panel A, the mycoplasmal proteins (0.2 μg, lane 1) can be detected unambiguously with the untreated antiserum. However, the lambda lysate (40 μg, lane 3) contributes a broad smear and two bands at approx. 90 and 33 kDa. The total protein of *E. coli* (40 μg, lane 2) can be seen as a 90 kDa band and three minor bands at approx. 64, 44 and 35 kDa positions. Absorption of antiserum with the freeze-thaw-sonication extract (panel B) as well as the boiling extract (panel C) improved the screening specificity to the extent that the smear and cross-reacting bands were reduced but were still high enough to complicate the immunoscreening

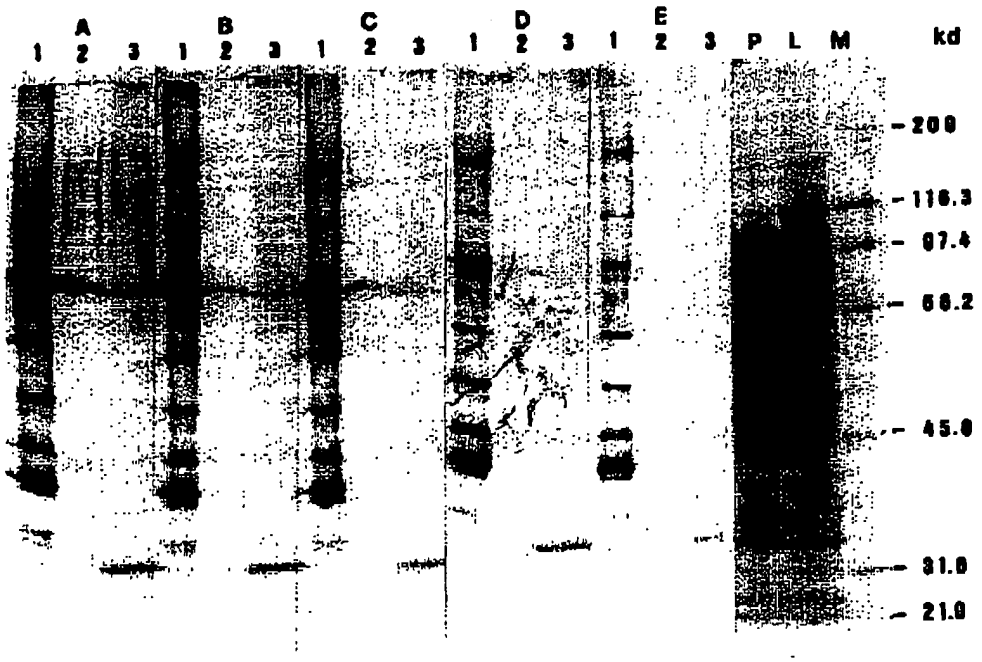

Fig. 1. Immunoscreening of surface protein genes of *M. hyopneumoniae* with antiserum purified by various absorption methods. The effects on immunoscreening were performed by a Western blot analysis as described in the text. Each antiserum preparation (panels A–E) was diluted 1:1000 with TBS, then reacted with the blotted NC paper bearing (lane 1) total proteins of *M. hyopneumoniae* strain 232, 0.2 μg; (lane 2) total proteins of *E. coli* P2392, 40 μg; and (lane 3) *E. coli* P2392 lambda lysate proteins, 40 μg. Lanes P, L, M and panels A–E are defined as follows: (P) total proteins of *E. coli* P2392, 40 μg; (L) Coomassie blue staining of *E. coli* P2392 lysate (by λEMBL3), 40 μg protein; (M) Protein size marker (Bio-Rad), 200 kDa, 116.3 kDa, 97.4 kDa, 66.2 kDa, 45.0 kDa, 31.0 kDa and 21.0 kDa. (A) Immunoscreening with untreated antiserum. (B) Absorption with *E. coli* P2392 treated with freeze-thaw-sonication process. (C) Absorption with boiling extract of *E. coli* P2392. (D) Absorption with both sonicated and boiling extracts of *E. coli* P2392. (E) Repeated absorptions with both sonicated and boiling extracts of *E. coli* P2392.

process. When the antiserum was absorbed with the sonicated and boiling extracts together, the background noise was reduced drastically, as shown in panel D. Dual treatments with the two extracts, as shown in panel E, improved the specificity even further so that only the cross-reacting 35 kDa band of the lambda lysate was left, while the mycoplasmal proteins were still clearly visualized.

The present study has demonstrated that a simple combination of the sonicated and boiling extracts of the host cells can greatly reduced the unwanted nonspecificities of the antiserum and should contribute to a successful immunoscreening process. This rather simple and efficient method should be applicable to most of the antiserum purification and immunoscreening processes. The more traditional antiserum purification methods, which include the absorption of the host protein coupled to solid supports, and affinity purification methods, which require harsh

denaturing condition for the dissociation of bound antibodies, seem to be less attractive. The criterion of the present antiserum purification method could be the feasible removal of the cross-reactive intracellular and membrane proteins by sonication extract and boiling extract, respectively.

4. Acknowledgement

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HANDBOOK OF EXPERIMENTAL IMMUNOLOGY
IN FOUR VOLUMES

Volume 1: Immunochemistry

EDITED BY

D. M. WEIR MD, FRCP
Professor of Microbial Immunology, University of Edinburgh, Scotland

CO-EDITORS

L. A. Herzenberg PhD
Professor of Genetics, Stanford University, USA

Caroline Blackwell PhD
Lecturer, Department of Bacteriology, University of Edinburgh, Scotland

Leonore A. Herzenberg DSc
Senior Research Associate, Department of Genetics, Stanford University, USA

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Chapter 8
Immunization of experimental animals

D. W. DRESSER

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Some basic procedures, 8.7
Preparation and use of three common adjuvants, 8.9
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This chapter is intended as a practical guide and is neither a comprehensive review of immunization procedures nor is it an account of recent researches into the nature and action of adjuvants. In so far as immunization procedures are concerned, there has been little progress in the thirty years since Kabat & Meyer [76] pointed out the 'arbitrary' nature of these procedures and Cohn [29] observed that they were 'the least developed phase of immunology'. This situation probably arises because of an understandable dearth of systematic studies, a somewhat haphazard attitude to antisera by many workers, and a remarkably efficient oral tradition for the dissemination of satisfactory protocols of immunization methodologies. Consequently these circumstances, together with a lack of space, compel this chapter to be a personal and idiosyncratic account of immunization procedures suitable for use with experimental animals.

The study of non-specific potentiators of immune responsiveness (adjuvants), as a result of recent advances in chemical and biological expertise, has in contrast become a precise and rapidly progressing science. For those interested in pursuing this subject there are several recent reviews on the nature and action of adjuvants. Chemical approaches to the structure and synthesis of molecules with adjuvant properties have been comprehensively reviewed by Jolles & Paraf [74], Whitehouse [136], Lederer [87], and Parant [105], while the other side of the coin, i.e. adjuvants seen from the biological point of view, is discussed in a Ciba Symposium [139] and in recent reviews by Waksman [133] and Bomford [16].

This chapter will be divided into two main sections. The first will be a simplified account of the immune response, fleshed out by somewhat more detailed consideration of aspects which are directly relevant to quantity and quality of antibody. In this section controlling mechanisms such as antibody feedback and acquired immunological tolerance will be considered in their role as antagonists of high titre antisera. The fact that antibody levels in excess of 50 mg/ml serum can sometimes be obtained [81,7] illustrates that in some instances the immune system can be turned in a single direction and partly relieved of those controls which normally maintain antibody levels at a more physiological level (1–5 mg/ml). The second main section will be a cookery book account of a few popular recipes together with a brief discussion of variations in procedure which have in certain circumstances been found to be useful. Although Migita & Matsuhashi [96] have catalogued most of the relevant literature to date, and there are excellent recent reviews of immunization protocols for use in experimental animals [25,54,138], there do not appear to be any recently published systematic studies of immunization procedures in any species; this section is therefore an entirely subjective amalgam of the author's own experience and that of a large number of colleagues who have been generous in their help.

The immune response

An immune response is the consequence of a complex sequence of events involving antigen and at least three kinds of lymphoid or reticuloendothelial cells. Most antigens require T cell help (T_H) to stimulate B cells into antibody production (TD or thymus-dependent antigens), while others, highly polymerized polysaccharides in particular, do not require such help (TI or thymus-independent). Both kinds of antigen may require the involvement of a third kind of cell, a macrophage-like antigen processing and presenting cell [67], which to avoid unnecessary complication at this point will simply be called an accessory (A) cell. A further complexity is introduced into the system by the different classes (isotypes) of antibody molecule (immunoglobulin) produced by B cells and the past immunization history of the animal which is reflected by the number of B memory cells present. A high number of memory cells results in a very large and much more rapid response to a second injection of

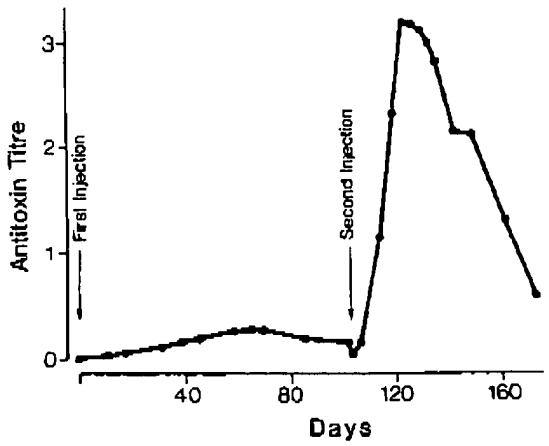


Fig. 8.1. A classical primary and secondary response of a rabbit to diphtheria toxoid. Adapted from Glenny & Sudmersen [55].

antigen—a secondary response· a classical feature of the immune response (Fig. 8.1) [24].

It is clear that the nature of the antigen introduced parenterally into an animal is important; however, the amount is of even greater significance, as will be seen later—too little or, worse, too much antigen can be strikingly counter-productive from the point of view of the immunologist trying to obtain a maximum yield of antibody. Apart from these general considerations relating to efficient immunization, there are several general points of relevance. Immune responsiveness is strikingly age dependent [78,93], newborn mammals having next to no innate ability to synthesize antibody and old animals showing a response very significantly reduced by comparison with that seen in their prime. A further feature of the newborn mammal is that in most species maternal antibody is transferred passively either across the placenta or orally through the colostrum or milk [20]: such passively acquired antibody can interfere with the process of active immunization. Certain antigens (often synthetic polypeptides) can elicit an immune response in one inbred strain but not another. This ability to respond has in many cases been shown to be under genetic control of loci closely linked to or part of the major histocompatibility complex (MHC) [9]. Similar genetic factors are undoubtedly a major source of variation in an immune response when outbred animals are immunized. However there must be additional sources of variation since considerable differences can be observed among animals of the same inbred strain. Finally, the general health of the animal being used to raise an antiserum can have a significant effect on antibody levels. Poor nutrition, disease ridden animals or dirty housing [58] and other stress situations [79] can be reflected in poor responses.

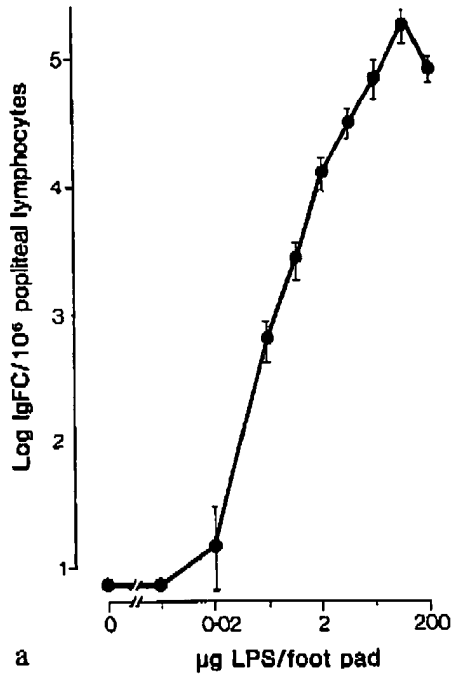
It is now possible to state the first general guidelines for immunization:

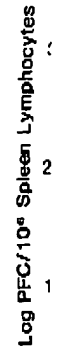
1 *use healthy unstressed young adult animals*;

2 *use several individuals*—due to genetic and other sources of variation a significant proportion of animals immunized may be poor- or non-responders, as few as 10% of rabbits immunized with a peptide hormone make a useful antiserum [14];

Antigen dose

The effect of different doses of antigen on an ensuing immune response is both of theoretical interest and of paramount practical importance. Fig. 8.2a–c illustrates typical dose–response curves for *in vivo* and *in vitro* responses: all show a sigmoid curve with a maximum. Presumably the most efficient point on this curve is the dose of antigen which gives the most antibody at the highest possible rate in terms of moles of antibody per mole of antigen. Since it is likely that it is the dose actually reaching the appropriate parts of the lympho-reticular system which matters, this point of maximum antigen efficiency may in practice be affected by the route of administration but perhaps





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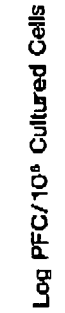

Fig. 8
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RBC

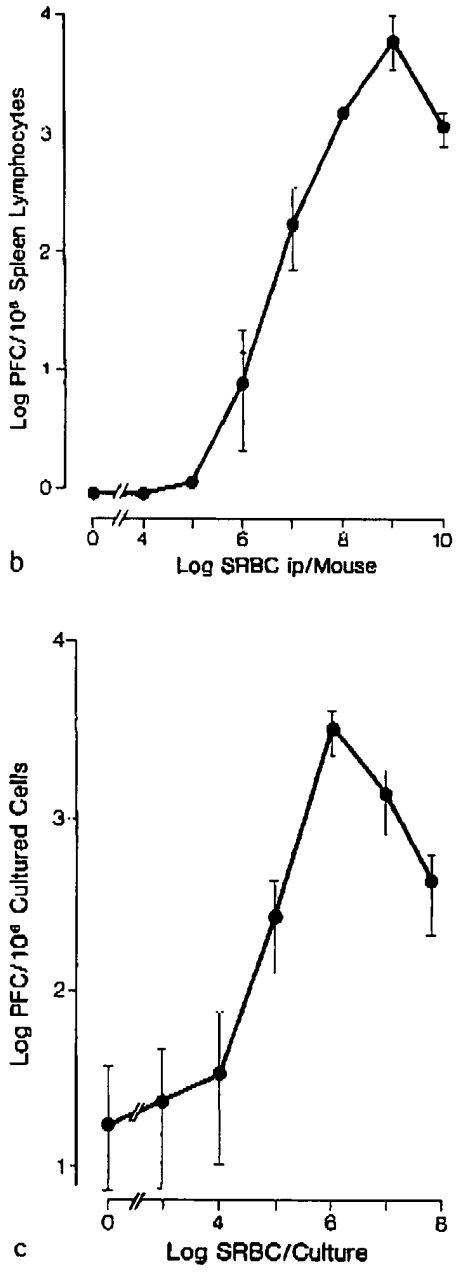

Fig. 8.2. Three IgM responses to a range of antigen or mitogen doses: (a) LPS injected into footpads of CBA mice—IgM PFC; (b) sheep RBC injected i.p. into CBA mice—IgM PFC; (c) spleen cells from CBA mice injected i.p. 3 months previously. 10^7 cells/ml in Mini-Marbrook chamber [92] together with a range of doses of sheep RBC—IgM PFC after 5 days of culture [88].

also by the stability of the antigen in the immunized animal. For example a small dose of antigen injected into a site with discrete drainage into a single lymph node (LN), such as the hind footpad—popliteal LN or the peritoneum—parathymic LN systems [117], may show an antigen dose maximum for the response in the local draining LN, which is disproportionately less than required to reach a maximum response for the animal as a whole. It will be clear, therefore, that measuring responsiveness in terms of antibody-forming cells (AFC) in an individual lymph node will probably give a different response curve to that obtained from serum antibody levels. The numbers of AFC in the spleen after intraperitoneal (i.p.) or intravenous (i.v.) injection of sheep erythrocytes into mice seem to reflect humoral antibody levels fairly closely, and show that the intravenous route is about ten times more efficient (i.e. needs tenfold less antigen) than the intraperitoneal route [141]. It has also been shown that small doses of SRBC injected into the mesenteric vein were ineffective by comparison with the same dose injected into the tail vein (unpublished data), presumably because the antigen was ingested by liver macrophages and never reached the antigen-presenting cells in any of the lymphoid organs.

The isotypes of responding antibodies may reflect differences in route of infection or immunization. For example there is a well-recognized association between immunization through the 'gut route' and a largely IgA response [12]. With a few exceptions such as oral immunization by attenuated poliomyelitis virus (Sabin), the effect of route of immunization on the development of cytotoxic T cells in the human subject [95], or experimental induction of immunity to influenza virus through inhalation by mice [142], these non-parenteral routes are unlikely to be of much interest to workers simply interested in raising antisera.

Unless one is either exceptionally lucky or unusually skilful, most antigen preparations are contaminated by minor components. Fig. 8.3 illustrates a hypothetical dose response to an antigen preparation containing a 1% contaminant of similar immunogenicity to the major component. It can be seen that at very low doses the response is entirely directed to the major component and at the higher doses the response to the minor component might even exceed that to the major component [30]. Antigen economy reinforces specificity for the major component of a mixture. However, should an antiserum be required which 'sees' the minor components, e.g. when an anti-'whole serum' reagent is being made, then repeated immunization with large doses of the antigen mixture will be called for.

Crude antigen dose–response relationships in them-

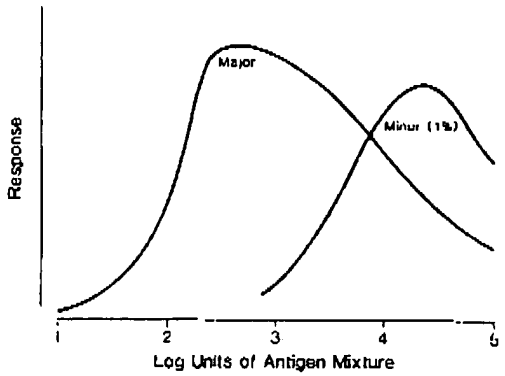

Fig. 8.3. A hypothetical dose-response to an antigen mixture which illustrates that high specificity of the response to the major component is obtained at the low end of the effective dose range. Good responses to the minor components are seen at doses which are supraoptimal for the major component.

selves say little that is really informative about the concentration of antigens in the immediate environment of the cells participating in the humoral response. Siskind has demonstrated that the net affinity of antibodies synthesized in a response falls as the dose of inducing antigen is increased. This is interpreted in the light of the clonal selection theory [72,127,23] as an example of competition for antigen by the immunoglobulin receptors of precursor B cells: at low antigen concentration only the cells with high affinity receptors can bind enough antigens for long enough to be 'turned on' [121,124]. It is understood by this and all other clonal-selection-based hypotheses that the receptors on a B cell are a precise sample of the immunoglobulin molecules which that cell and its direct descendants will be capable of synthesizing and secreting. Therefore this introduces the concept of a threshold concentration of antigen required to be maintained for a sufficient time, for immune induction to take place. Sequestration in sites irrelevant to the cells of the immune response, and catabolic breakdown both provide examples of natural mechanisms which will tend to reduce the effective concentration of antigen. Table 8.1 summarizes the result of a simple experiment [107] which shows that a dose of antigen, apparently too small to stimulate a secondary immune response, can be made powerfully immunogenic, simply by dividing the dose and spreading the injections over a few days. A similar potentiation of a primary response to sheep erythrocytes has been observed using higher total doses (4×10^5) but with the same timing of the injections. Maintaining the antigen concentration above such a threshold for sufficiently

Table 8.1. A comparison between a single and four divided doses of antigen

	Response*	
Sheep erythrocytes (i.p.)	IgM	IgG
4×10^4 on day 0	15	6
1×10^4 on days 0, 2, 4, 6	173	77

* Arithmetic mean integrated response—sum of PFC on days 5–21 × 10^4—four CBA mice per group per time point. 10^7 'cloned' primed spleen cells adoptively transferred (i.v.) to irradiated syngeneic recipients on day 0.

long may be the basis of action of the so-called depot adjuvants (to be mentioned again in the section on immunopotentiation), and of the success of immunization protocols which advocate the repeated injection at short intervals of small doses of antigen.

Control of the immune response

It is a practical axiom that immune responses are self limiting and, with certain rare and unusual exceptions [7,81], are in the long term controlled at relatively low levels of circulating antibody. The overall effectiveness of immunity to infection lies in the development of memory cells after a first exposure to antigen (primary response): these memory cells are the basis of the secondary response [23], which enables an animal to respond to a second exposure to antigen by a much more rapid, massive, and perhaps more avid response than previously.

Since a specific immune response is driven by the presence of antigen, catabolic destruction of antigen after ingestion by certain types of macrophage may of necessity comprise the first line of defence and perhaps one of the most effective controls of the immune response in the natural state. However, it became clear in the early days of immunology that although repeated injections of antigen can increase antibody levels in the serum, sometimes to quite high levels, a point is reached when no amount of antigenic stimulation will lead to further increases. Other control mechanisms including antibody feedback, cell mediated suppression, and acquired immunologic tolerance may be involved. Each is relevant to procedures for successful immunization.

Specific passive immunization immediately prior to the injection of antigen has been shown to be inhibitory to both primary and secondary responses [112,131]. However, in certain circumstances Ab-Ag

complexes may actually potentiate responsiveness [82,129]. The large amount of antibody present may act by reducing the effective concentration of antigen by accelerating clearance of antigen and perhaps by competing with B cell receptors for antigen within the lymphoid organs. Whatever the mechanism, this kind of experiment illuminates a phenomenon observed *in vivo*, namely that giving an animal a boost injection of antigen, when there is still a high antibody titre from a previous injection of the same antigen, is often quite ineffective.

Antibodies of one class (IgG) can influence the production of antibodies to the same antigen but of another class (IgM) in a similar feedback manner [60,113]. In contrast to these examples, antibody feedback control may be mediated through antibodies specific for parts of the immunoglobulin molecule itself, e.g. through isotypic [31], allotypic [36,63,64,91] or idiotypic determinants [73,132]. In recent years the 'network' hypothesis has focused attention on the latter both from the point of view of control of 'individual' antibodies but also the development and maintenance of the B cell repertoire which enables an animal to be capable of responding to an immense range of antigenic determinants other than the great majority of those represented in its own internal environment. This is the principle of Ehrlich's *Horror Autotoxicus* now enshrined in the theory of *self* and *not self* discrimination [24] and the process of acquiring a state of tolerance to self determinants [13,100].

In so far as this chapter is concerned, tolerance induction, being the specific antithesis of antibody production, is to be avoided. It is of some importance, therefore, to know of some of the conditions which favour the one or the other. At the cellular level tolerance may be effected by one or both of two possible mechanisms: (1) the elimination of precursor B (or T_H) cells with specificity for the determinants in question (clonal elimination, clonal abortion, etc.) [104]; and (2) the induction of a (antigen or possibly idiotype) specific cell-mediated suppression by certain T cells (T_S), which specifically control the differentiation or even the elimination of precursor B cells [64]. Whatever the cellular mechanism of tolerance induction, it seems highly likely that any situation in which antigen can be maintained in the body at a sufficient concentration (10^{-10}–10^{-8} mol/l) for sufficiently long (several days) will result in a state of tolerance—the animal is refractory to antigen even in its most immunogenic forms [42]. Thus it seems relevant at this point to list circumstances which favour tolerance induction and which are, therefore, antithetic to the production of antisera. These are: (1) supraoptimal doses of antigen · already discussed; (2) immaturity of the animal—the fetus has little if any ability to synthesize its own antibody although this capacity is rapidly acquired in the weeks immediately after birth [78,93]; nevertheless it is clear that the immune mechanism of the newborn is sufficiently developed to be highly susceptible to the induction of tolerance [13]; (3) immune responsiveness of the adult can be non-specifically reduced by treatment with ionizing irradiation [126], drugs [122] or antilymphocyte serum (ALS) [85]; combination of suppressive treatment and injection of antigen can lead to (partial) tolerance [116]; non-immunogenicity of an antigen—this may seem to be a contradiction in terms but antigenicity is taken here to mean the ability of a particular configuration on a molecule (antigen) to be specifically bound by the specific site of an immunoglobulin (antibody). Immunogenicity refers to the ability of that antigen to simulate antibody production, although other authors have somewhat different views [33,80]. An antigen can be non-immunogenic because it is already tolerated due to prior and appropriate exposure to the immune mechanisms or because it has an intrinsic property which renders it 'invisible' to the unprimed immune system. Various xenogeneic and allogeneic immunoglobulins are an example of protein molecules which can in certain animals lack intrinsic immunogenicity [38], possibly because they cannot bind to accessory cells [32]. In general, weakly (non)-immunogenic antigens are soluble, monomeric, and lack binding affinity for the cellular constituents of the recipient animal. The insolubilization by adsorption of such an antigen on to inert particles (alum, bentonite, beads of synthetic polymers) or aggregation (cross-linking reagents, heat treatment, precipitation by antibody [82,129]) can render such an antigen highly immunogenic. Similarly a monomeric immunoglobulin in solution, otherwise non-immunogenic, can be highly immunogenic if it is itself an antibody to one of the cellular constituents of the immune response [84,40]. As will be seen in the next section, the concommitant injection of an 'adjuvant' will also convert a weakly immunogenic antigen into a strongly immunogenic one; a possible implication is that the antigen binds to B cells which then require a non-specific stimulus from T or A cells to convert the antigenic signal at the surface of the B cell from tolerogenic to immunogenic [41]. This is probably all a gross simplification but will serve to emphasize the basic message of this chapter, i.e. ways of increasing immunogenicity in the production of antibody and immunity.

It is now possible to state some more guidelines for immunization as a summary of the two previous sub-sections:

3 use as little antigen as is practicable.

4 *maximize immunogenicity*—avoid inducing tolerance; facilitate the targeting of the antigen to the appropriate accessory cells by injecting i.v. or i.p. or in several i.m., i.d. or s.c. sites;

5 *be patient*—wait until the antibody titre from the previous immunization has subsided before repeating an immunization and so avoid the effect of antibody feedback. Allow sufficient time for a population of B memory cells to develop [34].

Immunopotentiation

Immunopotentiation by increase in immunogenicity of an antigen molecule has been mentioned in the previous section. However, it is also possible to stimulate 'immunity' by: (a) manipulation of the mechanism for T-B cell co-operation (the carrier effect); (b) the use of a third-party substance which, when injected at about the same time as antigen, can have a potentiating effect on immune responsiveness (adjuvant) [19,74,136,139]; and (c) manipulation (through phenomena such as the allogeneic effect [77].

A variety of T cell (T_H) helps B cells to respond to a certain type of antigen (TD: T-dependent) [27]. The antigenic determinants on the macromolecular antigen recognized by the helper T cell are different from those recognized by the B cell [98,109]. Since this is usually demonstrated in a hapten-carrier system [86], this aspect of T-B co-operation is sometimes called the 'carrier effect'. The response to a particular small-molecular-weight determinant (hapten) may be potentiated by first priming an animal with a suitably immunogenic carrier, e.g. a macromolecule such as keyhole limpet haemocyanin (KLH), chicken ovalbumin (OA) or bovine serum albumin (BSA). After the animal has been rested (guideline 5) the required hapten is (covalently) coupled to the carrier and injected. Often the response to the hapten is considerably better than if the animal had not been carrier primed. It will be obvious that this methodology will be most suitable for the preparation of antisera to small molecules (hormones, other peptides, etc.). It should be noted that whether or not the carrier effect is used in this way, molecules to be used for inducing antibody production which have a molecular weight less than about 3000 must be coupled to a macromolecular carrier and those less than six to ten thousand probably should be so coupled.

It is unlikely that all adjuvants act in the same way, and they may do so in one or more of up to six or seven possible modes of action. These can be summarized as follows.

(1) They can act as a differentiational stimulus or signal which induces a B cell precursor to enter a path leading to immunity rather than tolerance; they create a situation where a soluble antigen becomes immunogenic [41].

(2) They may provide a stimulus which enhances post-antigen proliferation (burst size), the mechanism whereby a single precursor B cell divides to form a clone of antibody-secreting cells [45]. This mechanism is likely to be most significant for antigens which possess their own strong intrinsic immunogenicity.

(3) Spring a lymphocyte 'trap' which leads to circulating lymphoid cells (> 80% T cells) being temporarily sequestered in local lymph nodes draining a site of antigen adjuvant injection, thereby increasing the chance of a favourable interaction between B, T, and A cells and antigen [128,46,53]. This may be related to the formation of disseminated granulomata and ultimately to a granuloma at the site of injection; the degree of humoral immune responsiveness has been shown in at least one experimental system to be directly related to the presence of granulomata arising in response to the tubercle in adjuvants of the Freund type [125].

(4) Some adjuvants may alter the ratio of T_H to T_S in a given population, thereby increasing or decreasing responsiveness to TD antigens.

(5) The depot effect, which is seen when antigen leaches slowly from a water-in-oil emulsion of a Freund-type adjuvant, may mimic the continuous release of antigen during the early part of a natural infection [51,52]. It seems likely that humoral immunity has evolved to cope with, and is therefore well adapted to, such a form of antigenic challenge. As mentioned in an earlier section, at lower doses of antigen (SRBC) four divided doses at 2-day intervals are far more effective than the same total dose injected at one time.

(6) The action of some adjuvants, of which *Bordetella pertussis* (Bp) may be a good example, could be more apparent than real, in that they may enhance the sensitivity of the read-out (histamine sensitivity), rather than lead to increased antibody production [94].

The study of the nature of adjuvants, starting from an analysis of the active fractions in tubercle and gram-negative bacterial endotoxin [89], has led to the commercial availability (Inst. Pasteur) of synthetic adjuvants. Muramyl dipeptide (MDP), when added to a tubercle free Freund-type adjuvant (FIA), can enhance immune responsiveness to levels close to that obtained with Freund complete adjuvant (FCA) [87,105]. Oil-based adjuvants of the Freund type are quite unsuitable for human use [137]; it is important that there should soon be development of well defined and characterized adjuvants with no unfortunate side effects such as abscesses, or the induction of one or more of a multitude of autoimmune disorders. The synthetic adjuvants are a promising line of research in this area. However from the point of view of the research immunologist wishing to raise high titre antisera it appears to the author and all those colleagues consulted that there is no adjuvant which

can exceed the efficacy of Freund complete adjuvant. Some adjuvants may be very nearly as good or more convenient to use (alum-precipitated antigen plus Bp), or in certain circumstances they may have less unfortunate side-effects on the injected animal. Howevers for sheer all round reliability FCA/FIA should be the first choice.

Notwithstanding its obvious virtues, FCA does have its blacker side. It is unsurpassed as a means of inducing autoimmunity [137,100] to certain 'self' antigens which at first sight one might have thought would have induced a state of natural tolerance. However some of these antigens may in a normal healthy animal never come into contact with the lymphoid system and rely in part for their functional non-immunogenicity on a barrier to exclude peripatetic lymphocytes from primary contact (lens, CNS) or to an innate lack of immunogenicity of healthy tissue or products (thyroglobulin, CNS [37]). It is only in a pathological situation, in animals injected with FCA, or more certainly when mixed in FCA, that these antigens become immunogenic and exposed to the immune mechanism, leading to a state of autoimmunity [100]. It is this, together with a tendency for the development of sterile abscesses in some species (including human) which makes Freund adjuvant totally unacceptable for human use [137].

Other recent lines of research on adjuvants which hold promise for future use in animals and perhaps eventually in man, are natural substances with preference for different cellular compartments, e.g. lipopolysaccharide (LPS) which possibly affects B cells directly or may do so through a potentiating effect on helper (T_H) but not suppressor (T_S) cells [43,4]. Lentinan (a natural 1-6 glucan originating from the basidiomycete *Lentinus edodes*) is thought to stimulate T_H [43,90]. Saponin seems to be another substance which may become an acceptable adjuvant [15], either injected as a mixture with antigen or incorporated into liposomes. Liposomes, which are made from artificial membrane vesicles, can be prepared so that they contain water-soluble antigens, and by virtue of their size and surface properties can be efficiently targeted to the macrophage [2,130]. Incorporation of Lipid A (extracted from LPS) into the artificial membrane can greatly increase the efficacy of these liposomes [103]. More refined targeting for antigens or drugs, imparted to these artificial membranes by virtue of the incorporation of suitably prepared specific antibodies (mIg), may be an exciting prospect for the near future.

The triple vaccine introduced by Ramon (tetanus toxoid, diphtheria toxoid adsorbed onto alum, plus Bp) is the only example of a *de facto* adjuvant for human use. Although the pertussis was introduced as an antigen it almost certainly has immunopotentiating properties together with a small but finite risk to autoimmune neurological complications (post-pertussis encephalitis) (see ref 28 for a detailed discussion of the techniques and ethical considerations of immunization of the human subject). Antigen adsorbed onto alum (aluminium phosphate/sulphate) together with Bp is a powerful adjuvant mixture when injected intraperitoneally into rats and mice [39]. An interesting side issue which throws a little light on the mechanism of adjuvants, is the phenomenon of 'immune deviation' [135,10,6]. Mice and guinea-pigs injected with alum-adsorbed antigen make an almost exclusively IgG_1 response. Further boosting with antigen in FCA leads only to further IgG_1 responses. *De novo* immunization with antigen in FCA leads to an IgG_1, IgG_2, and a delayed hypersensitivity response. Is this a possible example of isotype preference perpetuated by positive antibody feedback?

The final guideline:

6 *FCA/FIA is unsurpassed as an adjuvant and depot*, but be careful when using it (see note on safety on page 8.15).

Some basic procedures

Weakly immunogenic antigens

Example 1. Immunizing a rabbit with mouse IgG

FCA

It is assumed that a suitable purified mouse IgG (myeloma or hybridoma) has been acquired or prepared, perhaps using one of the methods outlined in Chapters 108 and 111). For each rabbit take 0.25–0.5 ml of sterile solution containing 100–150 μg of protein, and emulsify this with an equal volume of Freund complete adjuvant (FCA: often abbreviated as CFA). A simple way of doing this and of making other preparations of antigens ready for injection is described later in this section. The rabbit is injected intramuscularly (i.m.) in one thigh with 0.5–1.0 ml of the emulsion. The injection is repeated 7–10 days later in the other thigh. The rabbit is now 'rested' for a minimum of 8 weeks—longer if practicable: rabbits rested for a year have been found to respond very well to a boost injection! The rabbit is boosted with a similar amount of antigen made up in Freund incomplete adjuvant (FIA), i.e. without the mycobacteria present in FCA. The boost injections should be made subcutaneously (s.c.) in 5–8 sites. Six to eight days later the rabbit should be bled and the serum tested for antibody activity by a standard method (Ouchterlony, Mancini, IEP, RIA, or ELISA etc.). If there is a good titre, bleeding should continue (twice weekly) until the

using a skin graft, although much more labour intensive, is especially suitable for weak H-2 differences. The mice should be bled 7 days after the last injection.

Sometimes it is possible to raise anti-MHC (H-2) antisera in congenic pairs of strains, and this has the advantage of avoiding responses to undefined or unsuspected alloantigens [115]. MHC congenic mouse (and rat) strains are widely available.

Neoplastic tissues including leukaemias, ascites, tumours, and homogenates of solid and normal tissues, as well as lyophylized tissue, can be used as a source of H-2 antigen. Similar techniques have been used to raise anti-MHC antisera in rats and dogs [134].

Differentiation antigens

There are many loci specifying cellular antigens whose expression is limited to cells with particular functions or to cells at certain points during their differentiation. Many of these loci are polymorphic so that their products can be alloantigens in appropriate individuals or inbred strains. Among the lymphocytes there are several well-defined examples, including Thy-1, Lyt, Lyb, TL, and PC, together with an unknown number of ill-defined or undetected antigen systems. It is for this reason that it is often best to raise antisera in pairs of (mouse) strains congenic for a particular alloantigen system [115]. For example Thy-1 occurs on T lymphocytes but not on B lymphocytes; Thy 1.1 occurs in AKR mice and Thy 1.2 in most of the other strains. There are at least two established congenic lines, in both of which Thy 1.1 (from AKR) has been back-crossed onto another inbred background (A/Jax and CBA/Ca). An alloantiserum can be raised, in either direction, by injecting mice of one congenic line six times i.p. with 10^7 thymocytes at one week intervals. The mice are bled 1 week after the last injection and the sera absorbed with liver cells and RBC to remove non-specific activity [111].

As an alternative, the protocol of Lake [83a] seems to be an excellent method for raising very high titre (IgM) anti-Thy-1 antisera. Mice are injected i.p. with a low dose (10^6) of washed allogeneic thymocytes and then rested for 3–6 weeks before being given two i.p. boost injections of a much larger dose (10^7) of the same allogeneic thymocytes at an interval of 1 week. Six or seven days after the second boost the mice can be bled or their spleen cells can be harvested 3–4 days after the second boost if the intention is to produce hybridoma lines.

Some Thy 1.1 determinants are shared with rats which can be used to raise anti Thy 1.2 antibodies after immunization with cells in FCA [48]. It is essential to realize that although the use of congenic strains is generally better than using inbred strains, it is not a panacea and absorption with various combinations of cellular antigens is still likely to be necessary. The specificity of each batch of antiserum should be checked carefully before use [118]. It is clear that the first move in the process of raising alloantisera against differentiation antigens would be to study the nature and availability of congenic strains. Many are available from commercial and other sources (Olac, Jackson Laboratories, etc.). The FASEB Handbooks [1] should be consulted.

Hybridomas

Many monoclonal antibodies (hybridomas) directed against cellular (allo) antigen systems have been developed. Some are available commercially and some can be obtained from the research workers concerned. Since all available hybridomas have not yet been codified or centrally indexed, it is necessary for an individual requiring such a reagent to consult the appropriate current literature, in addition to contacting one or more of the following sources of *monoclonal antibodies* (hybridomas) or the cell line synthesizing them:

American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852 (Catalogue available);
Monoclonal Bulletin (UK) Ludwig Institute for Cancer Research, Clifton Ave., Sutton, Surrey, U.K.

In addition, several commercial companies now supply monoclonal reagents—see current numbers of immunological journals (including *Immunology Today*) for relevant advertisements.

Minor H-loci (including H-Y)

Despite past reports apparently to the contrary, it now seems clear that in mice, of all the known histocompatibility (H-) loci, the H-2 is unique in that it elicits a humoral (B cell) as well as a CMI (T cell) response [119]. In this connection the H-Y locus provides two examples which illustrate points of importance to anyone wanting to raise antisera against cellular antigens: (1) the nature of the humoral antibodies which at first sight appear to have anti-H-Y activity; and (2) the problem of the immunological 'non-responder', and how in certain instances this non-responsiveness can be by-passed.

Antisera raised against 'H-Y' are now believed to react to a serologically defined male (SDM) product of a gene which is not on the Y-chromosome, while the evidence is consistent with the structural gene controlling rejection of male grafts by female mice, being on the Y-chromosome [120].

H-2^b strains of mice (C57Bl) are strong responders

to 'H-Y' antigens: 10^6–10^7 male spleen cells injected into syngeneic females by almost any route (s.c., i.p. or i.v.) will, after a single injection, stimulate a strong CMI (cytotoxicity) to H-Y antigen. However in non-H-2^b responder mice only a dose of between 5×10^6 and 2×10^7 spleen cells injected into the hind footpad, is effective. This is reminiscent of the phenomenon discovered by Yowell [115] in which H-2^b mice, apparently non-responders to hen egg-white lysozyme, become responders if injected into the hind footpads: this is an area rich in Langerhans' cells, a candidate for antigen-presenting cell *par excellence*. AKR and BALB/c are examples of strains which are total non-responders in the 'H-Y' system.

Anti-Idiotype (anti-Id)

In some instances these antigens are more difficult in the sense that greater persistence is often required, and the proportion of non-responding animals tends to be higher than with other antigens. Anti-Id will be considered under the headings of xeno-, allo- and syngeneic antisera.

Xenogeneic (1)

Antisera to idiotypic determinants on mouse, guinea-pig, and human immunoglobulins can be raised by the following procedure [123]. Fab fragment is prepared from a purified Ig preparation by standard enzyme digestion procedures. The Fab is bound to an immunoabsorbent column made with (rabbit or sheep) antibody to Fdμ of the same species as the Fab being prepared. After washing, some free antibody of the same specificity as that bound to the immunoabsorbent column is added to the fluid phase of the column. The Ab-Fab complex (4:1 by mass) is now eluted using thiocyanate in neutral buffer, the Ab and Fab dissociate but reform a complex on dialysis against saline. Ten to twenty micrograms of Fab (plus associated Ab) are emulsified in FCA and injected into a rabbit (or sheep) using the general protocol given in Example 1. The use of an Ab-Ag complex both facilitates handling of small quantities of protein and helps to potentiate the response. Satisfactory anti-Id sera can often be raised by immunization with Fab in FCA without the formation of a complex with an anti-Fab (Fd) antibody. This carrier-like potentiation can be achieved in other ways, e.g. the Fab (or Ig) can be coupled to a large and highly immunogenic antigen such as KLH or even LPS using glutaraldehyde (see '*syngeneic*' below) or some other suitable coupling agent. Some workers use prolonged immunization schedules for the preparation of anti-Id sera but this procedure should only be adopted when no immune response is elicited by the shorter schedule outline in Example 1: there is no merit in using more antigen than necessary.

Xenogeneic (2)

Guinea-pigs are injected i.v. with 5 mg of aggregate-free xenogeneic (rabbit) IgG. Immediately afterwards the animals are injected with 100 μg of affinity-purified (homogeneous) (rabbit) antibody in FCA in the hind footpads. Four weeks later the guinea-pigs are bled out. The i.v. injection of normal IgG of the same species as the affinity-purified antibody, seems to induce a state of tolerance or at least leads to a suppression of immunity to the majority of determinants and focusses the guinea-pig's (or rabbit's [83]) immune response on the unique (idiotypic) determinants of the antibody [49]. The method was originally used for raising antisera specific for mouse isotypes [59].

Allogeneic

It is possible that in some situations, allotypic differences between Ig immunogen and the immunized mouse can, by acting as a carrier determinant, potentiate the development of anti-Id antibody. However it seems more likely that the usual outcome of an allotypic difference will be to drastically out-compete the weaker Id determinants [18]. The 'carrier effect' (in a reversed sense), using non-allotypic differences, has been used to potentiate anti-Id responses in inbred mice. CBA mice sensitized to DNP by being skin painted with dinitrofluorobenzene (DNFB) were subsequently immunized with a mixture of *pertussis* and purified mouse myeloma (5563) coupled with DNP and then adsorbed on alum [71].

Anti-Id can also be raised in mice using the procedure outlined in Example 2. For example 100 μg of alum-precipitated myeloma protein can be injected with 2×10^9 *pertussis* organisms i.p. into mice of the same allotype as the myeloma protein: an allotypic (IgCH) difference between myeloma protein and mouse will, as mentioned above, result in a predominantly anti-allotype serum, q.v. If repeated (weekly) injections of alum-precipitated protein are to be used, small individual doses (5–10 μg) suffice and the *pertussis* should only be included in the first injection.

Syngeneic

The procedures used by Cazenave and colleagues [17] to raise anti-Id antisera are as follows: BALB/c mice are injected twice at an interval of 5 days with 75 μg of BALB/c IgA myeloma MOPC 460 (460Id) in FCA (s.c.). This is followed by 6 injections at weekly intervals of 75 μg MOPC 460 in saline. The BALB/c

mice 'see' the 460Id as a foreign determinant, perhaps because in normal circumstances this determinant is so rare that a state of natural tolerance is not induced. The antibody produced to 460Id is predominantly homogeneous and anti-(Id of (anti-460Id)) antibodies can be raised! Anti-460Id is affinity purified from an immunoabsorbent column of MOPC 460/Sepharose 4B. Pure antibody and keyhole limpet haemocyanin (KLH), both at a concentration of 0.5 mg/ml, are mixed with 0.05% glutaraldehyde until a definite opalescence forms. The reaction is stopped by adding lysine to a final concentration of 0.05 M. The conjugate is dialysed before being injected in FCA into more BALB/c mice, using the schedule outlined above for immunization.

Anti-immunoglobulin allotype

Anti-allotype sera can be raised in rabbits and mice using appropriate combinations of animals or inbred strains and one of the protocols outlined in Examples 1 and 2. Alternatively, complexes of antibody and bacteria can be used as powerful immunogens for this purpose. *Bordetella pertussis* (Bp), coated with anti-Bp raised in one strain (BALB/c), can be injected into mice of another strain (C57B1); the latter then, in addition to some anti-Bp antibody, also produce antibodies directed against the foreign allotypic determinants of the first strain [44]; in this example the C57B1 mice (Igh^b) produce an anti-Igh^a response. A similar procedure using *Proteus* bacteria (*P. morganii*) can be used in the rabbit. Details of the genetics of mouse allotypes, and methods for raising antisera to each of the six known polymorphic loci are given in Chapter 97.

Antisera to allotypic differences on chicken immunoglobulins have been raised by Ivanyi [69] using complexes of antibody and Bp in a manner analogous to that outlined above for mice. One milligram (4×10^9–4×10^{10}) of Bp coated with antibody is injected i.v. into 12-month-old chickens. These injections can be made at 2- to 4-week intervals for up to a year. With bleedings starting 1 week after the last injection, up to 50 ml of strong anti-allotype serum can be prepared from an individual bird.

Small molecules

To be immunogenic, small determinants (haptens) must be firmly coupled to a macromolecular carrier [86]. An example of the practical aspects of this principle is the preparation of antisera against peptide hormones. One protocol [14] employs the alternate use of two carriers (BSA and KLH), low doses of carrier conjugate, for example 0.1 nmol glucagon on 0.02 n mol BSA (~300 μg) are injected s.c. into rabbits in FCA. The injections of antigen in FCA are repeated at 1–3 month intervals until there is no improvement in titre, when a switch to the alternative carrier usually results in a significant boost in antihormone titre. As mentioned earlier (guideline 2), the number of animals which respond by producing an antiserum suitable for RIA may be as low as 10–15%: suitability in this context is not merely a question of titre but also implies that the antibody molecules are not damaged by iodination procedures and have specificity for the appropriate determinant.

This method can be used for many other small determinants as well as for somewhat larger molecules (such as insulin), which do not have an absolute requirement for coupling to a macromolecular carrier but whose immunogenicity is greatly increased if they are. Glutaraldehyde (see previous section) can be used as a coupling reagent: although simple to use it is not ideal because of the random nature of the cross-linking process. A more precise and elegant method involves the use of a suitable bifunctional coupling reagent which is commercially available from Pharmacia (Box A5, S-75104 Uppsala 1, Sweden, and local branches). (Leaflet: *SPDP heterobifunctional reagent*.)

Small quantities

Sometimes the immunologist ends a preparative procedure with extremely small amounts of a potential immunogen. There are several procedures which can be used in such a situation.

1 As mentioned earlier, on page 8.10, sheep can be immunized using as little as 1 μg Ig in 25 μl FCA injected intradermally [47].

2 Small doses and volumes can be injected directly into the popliteal lymph node or knee joint of the rabbit [65,83]. To facilitate the injection of 25 μl of FCA into the node the appropriate footpad is injected 6–18 hours before with 0.5 ml of lithium carmine which both enlarges and colours the node [102]. The injection should be made with a very fine needle (30-G) fitted to a glass syringe. The nodes can be reinjected 3 or 4 weeks later and i.v. boosts with soluble antigen made subsequently. As in previous examples the rabbits should be bled 7 days after the last injection.

3 Mice can be injected i.v. with small amounts of Ig (100 ng Bovine IgG) bound to bentonite particles (0.3–0.5 μm diameter) (see section on the preparation of bentonite particles—page 8.11). A procedure such as this may not result in high titre antisera but may be sufficiently potent to stimulate usable amounts or trigger sufficient cells to make the production of a hybridoma line a practical possibility.

4 Single bands can be cut from polyacrylamide gels

(PAGE), the protein electro-eluted for immunization in FCA or alternatively the antigen and gel can be frozen and thawed to break up the gel and the whole mixture emulsified in FCA ready for injection s.c. or i.m. The acrylamide gel seems to have adjuvant properties and high antibody titres have been obtained in rabbits immunized in this way [106].

Hybridomas

There are nearly as many variants on the theme of priming for hybridoma production as there are immunologists. Nearly all the methods for immunizing mice (or rats) discussed so far have been used. However they all have in common the recommendation that the best results are obtained if the spleen (or lymph node) cells are taken soon (2–4 days) after the last boosting dose of antigen, before humoral antibody production has reached its maximum. A further generalization is that it seems likely that the immunization procedures which produce the highest titre/avidity antisera are the best method for priming cells for hybridoma production. A final intravenous boost with antigen in solution seems to be highly effective in leading to the presence in the spleen of large numbers of primed cells which are suitable for fusion 3 days later.

Two alternative methods of giving cells a final boost before fusion have been found to be successful in certain circumstances.

1 Spleen cells from FCA-immunized mice can be adoptively transferred, together with soluble antigen, to irradiated syngeneic recipients. The spleens of these recipients are a rich source of primed cells suitable for fusion 4 days later [18,50].

2 Spleen cells primed *in vivo* can be placed in Dutton-Mishell [97] or Marbrook culture [92] with very small quantities of antigen. After 3 or 4 days, cells restimulated by antigen *in vitro* have been used successfully in the production of hybridoma lines [52,110].

See also under 'hybridoma' in two earlier sections: Example 2 (page 8.8) and 'Cellular alloantigens' (page 8.11).

Other species

So far the discussion has centred on the common laboratory species—rats, mice, and rabbits—with a few remarks about other species, such as sheep and guinea-pigs, made *en passant*. In this section it is intended that some examples and 'take-home' lessons learnt from other species will be discussed very briefly.

Guinea-pigs

These should never be injected more than once with FCA, if further injections must be made using antigen-in-oil emulsion, FIA should be used. Guinea-pigs also manifest the phenomenon of immune deviation (q.v.) in which immunization with an alum-adsorbed antigen stimulates an IgG_1 response and prevents (future) production of IgG_2 antibodies or DH. A first injection of antigen in FCA stimulated IgG_2, IgG_1, and DH (delayed hypersensitivity-CMI)—see page 8.7. These caveats apart, immunization of guinea-pigs is much as already described for rabbits and mice. When immunized with mouse Ig (mycloma) molecules in FCA, guinea-pigs tend to make a stronger class-specific (anti-Fc) response than a pan-specific (anti-Fab) response. The specificity of some antisera can be enhanced by the procedures outlined in *Xenogeneic (2)* on page 8.13.

Ungulates

In general, ungulates should be immunized by one of the protocols already described for the rabbit and sheep. Some care should be taken if i.v. boosts of antigen in solution are to be used since anaphylactic shock can lead to the loss of a valuable animal. An i.v. injection of a suitable antihistamine such as 'Phenergan' (Promethazine HCl), 5 or 10 minutes before the antigen, is advisable. A dose of 10 mg is suitable for a large rabbit. It should be remembered that some large animals are difficult to handle and may require a special crush or other restraint if they are to be injected and bled safely. Pigs are very difficult to bleed.

Primates

Many primates, including the human subject, tend to suffer from adverse effects (abscesses etc.) if FCA is injected i.m. or s.c. Since these animals are hyper-responsive and expensive, it is suggested that FCA is only used if, after due consideration, there appears to be no alternative. It may be possible to immunize primates by the method described for sheep where 25 µl of FCA containing a few micrograms of an antigen are injected at several sites intradermally (i.d.): the injection site should of course be inaccessible to the animal. In the sheep these injection sites scab over and eventually break to the exterior, which is likely to be uncomfortable for a while but leaves no permanent lesion. FCA and FIA should *never* be used in a human subject.

Note on safety

If there is a laboratory accident and someone stabs themselves with a needle being used for FCA, the

wound should at once be squeezed and if necessary enlarged with a clean scalpel blade just enough for it to bleed freely. It should then be scrubbed clean with soap and running water. If there has been a deep penetration accompanied by deposition of FCA, then medical advice should be sought immediately with a view to excision of the injected material. Some individuals can suffer from very unpleasant and prolonged effects as the consequence of the accidental injection of FCA [11,26,35]. Care should always be taken when injecting antigens in FCA, and this is especially so when these antigens are from the CNS (e.g. for the induction of allergic encephalomyelitis), and only workers experienced and skilled in these techniques should be allowed to make the injections.

Mineral oil (adjuvant) is used as a means of inducing multiple myelomas in susceptible strains of mice [108]. It may, therefore, be more than coincidence that Jules Freund died of multiple myeloma [3].

Fowls

Chickens can be immunized in a manner similar to that described in Example 5 for the rabbit. For example a large dose of antigens such as HSA and BSA (10–100 mg/kg body weight) is injected i.v. into the wing vein. A good primary response is seen 7–8 days later. A second i.v. injection of the same dose can be made a month later [70]. The rise and fall in antibody titre tends to be very sharp, and it is important to get the timing right. Chicken antibodies precipitate antigens best in high salt concentrations (1.4 M-NaCl).

Very much lower doses of antigen can be used if the birds are injected s.c. or i.m. with FCA [70]. For example 2–5 mg DNP-Bovine IgG in FCA injected i.m. using the protocol described in Example 1, results in a very high titre anti-DNP response. When injecting intact bacteria (*E. coli*) there is little difference whether FCA is used or not [140].

Bleeding

Techniques for animal handling and bleeding are described in Chapter 133. The author wishes to add only three points here.

1 Frequent, gentle handling of animals facilitates bleeding and injections, and may avoid the development of 'needle shyness'. Animals which are comfortable and relaxed, apart from being easier to handle, bleed more profusely than animals which are uncomfortable or frightened (Fig. 8.5). It is the author's experience that guillotine restraining boxes for rabbits are usually counter-productive and should only be used as a last resort.

2 A course of bleeds should be started at the peak of the humoral response and should be continued at regular intervals until the titre falls unacceptably low. Plasmaphoresis experiments [57] have shown that antibody titres return to a level close to that observed immediately prior to plasmaphoresis (or heavy bleeding [8]).

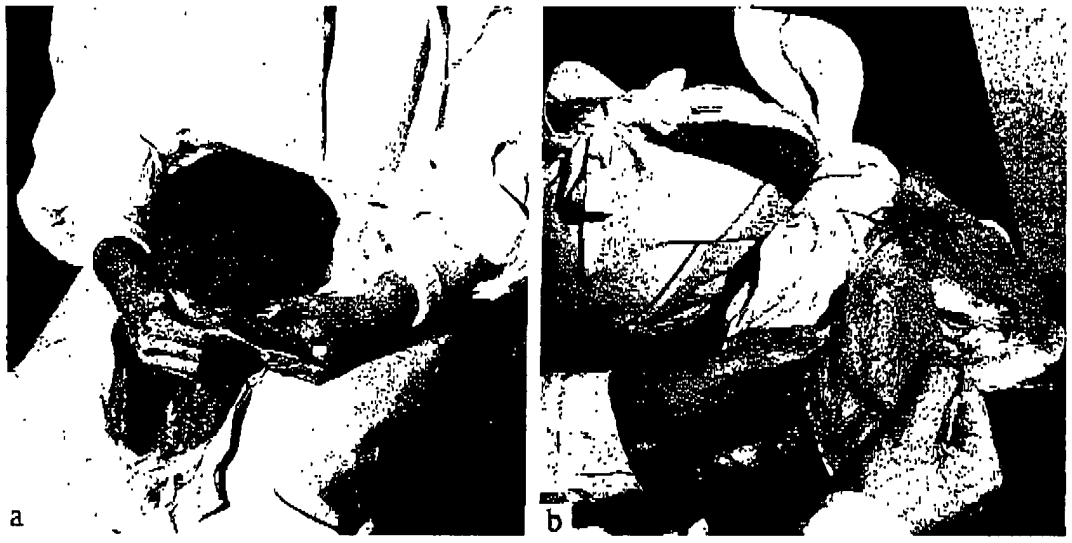

Fig. 8.5. Two ways of handling rabbits for bleeding from the marginal ear vein: (a) a rabbit which has become well behaved after regular handling; (b) a way of restraining a rabbit using a towel.

3 Experimental animals which are suffering any discomfort, for example from sterile or infected abscesses resulting from FCA injection, should be killed. If there is a positive titre, it would be sensible to bleed the animals out under total anaesthesia.

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Appendix I

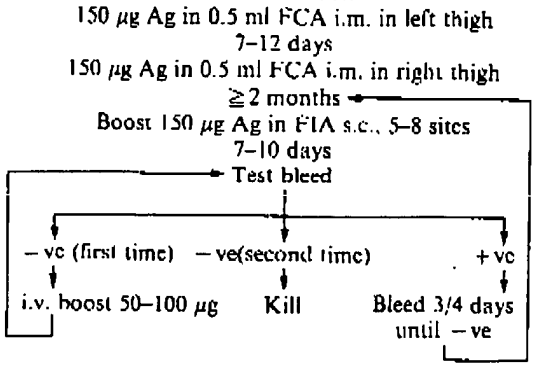
Good immunization guide: the guidelines listed in the text.

1 Use healthy, unstressed young adult animals.
2 Use several individuals.
3 Use as little antigen as is practicable.
4 Maximize immunogenicity.
5 Be patient.
6 FCA/FIA is unsurpassed as an adjuvant/depot.

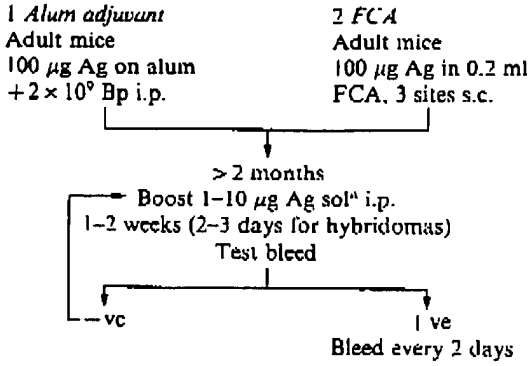
Appendix II

Summary of basic immunization procedures.

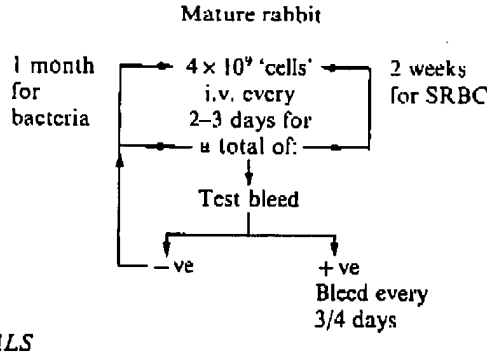
Example 1



Example 2

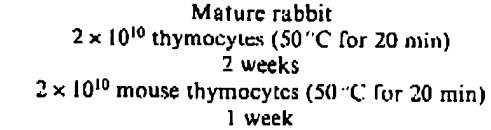


Examples 3 and 4

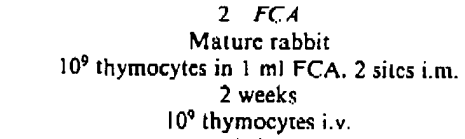


ALS

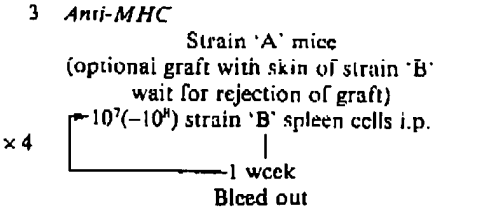
1 *No adjuvant*



2 *FCA*



3 *Anti-MHC*



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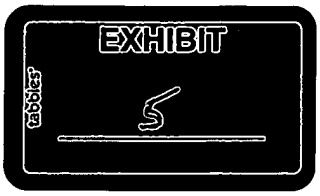

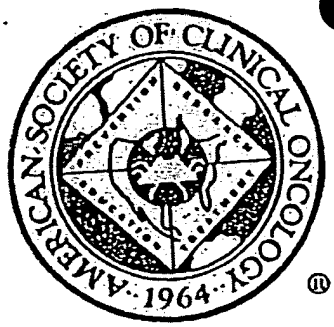

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Evaluation of a New Immunological Marker TGT (TURTEST®) in the Diagnosis of Lung Cancer. *A. Berlin, C. Chiaffitelli, V. Erkhov, V. Maximenko, I. Bakhlaev, E. Oleinik, A. Luongo. Dept. of Chemical Enzimology, Moscow State University, Moscow, Russia.; Center of Oncology and Radiotherapy, Rio Grande, Rio Grande do Sul, Brazil.; Dept. of Immunology, Hertzen Cancer Research Institute, Moscow, Russia.; Dept. of Clinical Oncology, Petrozavodsk State University, Petrozavodsk, Russia.; Dept. of Radiotherapy, University of Uruguay, Montevideo, Uruguay.*

The TGT (TURTEST ®) is an immunological marker based on a reaction of hemoagglutination by a specific anti-idiotypical, anti-embryonic serum. The TGT was developed in the Hertzen Cancer Research Institute (Moscow, Russia). To evaluate the validity of TGT in the differential diagnosis of pathological lung conditions, post-therapeutic follow-up and screening of population from 1994 to 1998 seven thousand six hundred and eighty seven (7, 687) patients from oncologic high-risk areas of Karelia (Russia), Montevideo (Uruguay) and Rio Grande do Sul (Brazil) underwent TGT. Differential diagnosis was studied with: 297 lung cancer (LUC) patients, 36 patients with benign lung tumor (BLT), 126 with non-neoplastic lung pathologies (NNLP) and 80 healthy patients. The sensitivity (S) observed according to the stage was: S (T1)=85.8%, S (T2)=90.6%, S (T3)=90.3% and S (T4)=87.5%, the average sensitivity was 88.6±2.3% and the average specificity (E) in healthy patients, BLT and NNLP groups was 90.0±5.9%. Post-therapeutic follow-up was performed with 160 LUC patients (TGT-positive) who had received radical surgery (RS) and 28 patients (TGT-positive) who had received non-radical surgery (NRS). In the case of RS (after 6 months) only 10.0% of the patients showed positive TGT, and in the case of NRS 72.0%. These results were used as a criterion of the effectiveness of the therapy. Screening of population: 6960 patients from high-risk areas were checked from 1994 through 1998. 204 positive results (2.9%) were obtained, 45 (22.0%) of which were diagnosed as having neoplasms in different locations right after the test was done (7 patients with LUC). 27.0% of these patients showed asymptomatic pathologies. The TGT is highly sensitive (S=88.6±2.3%) and specific (E=90.0±5.9%) to active malignant lung tumors. It could be used as a supplementary method in the screening and diagnosing of LUC, as well as to control the effectiveness of the chosen therapy and to monitor the progress of the disease.